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(54) Title: ANTAGONISTIC ANTI-AVB3 INTEGRIN ANTIBODIES			
(57) Abstract  Monoclonal antibodies that recognize the $\alpha_v\beta_3$ integrin receptor complex, but do not significantly bind to $\alpha_{IIb}\beta_{3A}$ , inhibit $\alpha_v\beta_3$ integrin-mediated diseases.			

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## ANTAGONISTIC ANTI-AVB3 INTEGRIN ANTIBODIES

This application claims the benefit of Serial No. 60/043,853, filed April 11, 1997.

## BACKGROUND OF THE INVENTION

## Field of the Invention

- 5 The present invention relates to antibodies which are useful as  $\alpha_v\beta_3$  integrin antagonists and as such are useful in pharmaceutical compositions and in methods for treating conditions mediated by  $\alpha_v\beta_3$  by inhibiting or antagonizing  $\alpha_v\beta_3$  integrins.

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## Background of the Invention

- Integrins are a group of cell surface glycoproteins that mediate cell adhesion and therefore are mediators  
15 of cell adhesion interactions that occur in various biological processes. Integrins are heterodimers composed of noncovalently linked  $\alpha$  and  $\beta$  polypeptide subunits. Currently at least eleven different  $\alpha$  subunits have been identified and at least six  
20 different  $\beta$  subunits have been identified. The various  $\alpha$  subunits can combine with various  $\beta$  subunits to form distinct integrins.

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The integrin identified as  $\alpha_v\beta_3$  (also known as the vitronectin receptor) has been identified as an integrin that plays a role in various conditions or disease states including tumor metastasis, solid tumor growth (neoplasia), osteoporosis, Paget's disease, humoral hypercalcemia of malignancy, angiogenesis, including tumor angiogenesis, retinopathy, arthritis, including rheumatoid arthritis, periodontal disease, psoriasis and smooth muscle cell migration (e.g. restenosis). Additionally, it has been found that such integrin inhibiting agents would be useful as antivirals, antifungals and antimicrobials. Thus, antibodies that selectively inhibit or antagonize  $\alpha_v\beta_3$  would be beneficial for treating such conditions.

15

It has been shown that the  $\alpha_v\beta_3$  integrin binds to a number of Arg-Gly-Asp (RGD) containing matrix macromolecules, such as fibrinogen (Bennett et al., Proc. Natl. Acad. Sci. USA, Vol. 80 (1983) 2417), fibronectin (Ginsberg et al., J. Clin. Invest., Vol. 71 (1983) 619-624), and von Willebrand factor (Ruggeri et al., Proc. Natl. Acad. Sci. USA, Vol. 79 (1982) 6038). Compounds containing the RGD sequence mimic extracellular matrix ligands so as to bind to cell surface receptors. However, it is also known that RGD peptides in general are non-selective for RGD dependent integrins. For example, most RGD peptides that bind to  $\alpha_v\beta_3$  also bind to  $\alpha_v\beta_5$ ,  $\alpha_v\beta_1$ , and  $\alpha_{IIb}\beta_{IIIa}$ . Antagonism of platelet  $\alpha_{IIb}\beta_{IIIa}$  (also known as the fibrinogen receptor) is known to block platelet aggregation in humans. In order to avoid bleeding side-effects when treating the conditions of disease states associated with the integrin  $\alpha_v\beta_3$ , it would be beneficial to develop selective antagonists of  $\alpha_v\beta_3$  as opposed to  $\alpha_{IIb}\beta_{IIIa}$ .

Antibodies selective for  $\alpha_v\beta_3$  offer such an advantage.

## SUMMARY OF THE INVENTION

The present invention involves murine monoclonal antibodies produced by hybridomas generated from mice immunized with human integrin  $\alpha_v\beta_3$ , or cells expressing  $\alpha_v\beta_3$ , that block the functional activity of  $\alpha_v\beta_3$ . More specifically, the present invention involves murine monoclonal antibodies, P113-7D6, P112-4C1, P113-12A6, P112-11D2, P112-10D4 and P113-1F3, which are complex-specific for  $\alpha_v\beta_3$  in the sense that they immunoreact with an integrin  $\alpha_v\beta_3$  complex and do not react with either of the integrin  $\alpha_v$  or  $\beta_3$  subunits individually. In addition, the inventive monoclonal antibodies do not significantly bind to  $\alpha_{IIb}\beta_{IIIa}$ ,  $\alpha_v\beta_1$ ,  $\alpha_v\beta_5$  or other RGD recognizing integrins. The monoclonal antibodies of this invention may also be used in a method of treating  $\alpha_v\beta_3$  integrin-mediated diseases or conditions by administering to a mammal in need of treatment an effective amount of an  $\alpha_v\beta_3$  integrin antibody that acts as an  $\alpha_v\beta_3$  integrin antagonist or inhibitor.

The monoclonal antibodies of the present invention can also be used in methods for delivering either cytotoxic or cytostatic compounds, nucleic and deoxynucleic acids or radioisotopes to cells having  $\alpha_v\beta_3$ -integrin. For example, the monoclonal antibodies of the present invention (or a Fab, Fab', F(ab')<sub>2</sub> and F(v) fragments thereof containing a paratope for  $\alpha_v\beta_3$ -integrin) can be conjugated to the cytotoxic or cytostatic compounds, nucleic acids or radioisotopes and then contacted with tissue or cells *in vivo* or *in vitro*.

## BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains one photograph executed in color. Copies of this patent with the color photograph will be provided by the Patent and

Trademark Office upon request and payment of the necessary fee.

Figure 1 shows that the purified anti- $\alpha_v\beta_3$  monoclonal  
5 antibodies P113-7D6, P112-4C1, P113-12A6, P112-11D2, P112-10D4, P113-1F3, LM609 (obtained from Chemicon) binds  $^{125}\text{I}$ -labeled  $\alpha_v\beta_3$  (panel A) but not  $^{125}\text{I}$ -labeled  $\alpha_{\text{Ib}}\beta_{\text{IIIa}}$  (panel B). The control monoclonal antibody, M399, binds neither  $^{125}\text{I}$ -labeled  $\alpha_v\beta_3$  nor  $\alpha_{\text{Ib}}\beta_{\text{IIIa}}$ . M399 is an  
10 irrelevant isotype matched control antibody produced by Monsanto Company. LM609 antibody was purchased from Chemicon (Tamecula, CA).

Figure 2 shows that monoclonal antibodies (50  $\mu\text{g/ml}$ )  
15 P113-7D6, P112-4C1, P113-12A6, P112-11D2, P112-10D4, P113-1F3, LM609, but not a control monoclonal antibody M399, inhibit Mn-induced binding of M21 (a melanoma cell line) cells to fibrinogen.

Figure 3 shows that the monoclonal antibodies (50  $\mu\text{g/ml}$ ) P112-4C1, P113-12A6, P112-11D2, but not the control monoclonal antibody M399, inhibit M21 cell (a human melanoma cell line) migration toward fibrinogen (Fg). This figure shows migration to Fg alone and Fg  
25 in the presence of antibody.

Figure 4 shows that monoclonal antibodies (50  $\mu\text{g/ml}$ ) P113-7D6, P112-4C1, P113-12A6, P112-11D2, P112-10D4, P113-1F3 and LM609 inhibit 293/B3, but not 293/B5 or  
30 293/B1, cell adhesion to vitronectin.

Figure 5 shows that monoclonal antibodies P113-7D6, P112-4C1, P113-12A6, P112-11D2, P112-10D4, P113-1F3, 23C6 and LM609 inhibit 293/B3 cell adhesion to  
35 vitronectin. M399 is a control monoclonal antibody. 23C6 antibody was purchased from PharMingen (San Diego, CA).

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Figure 6 shows that monoclonal antibodies (50  $\mu$ g/ml) P113-7D6, P112-4C1, P113-12A6, P112-11D2, P112-10D4, P113-1F3 inhibit rabbit osteoclast adhesion to osteopontin. M399 is a control monoclonal antibody.

5

Figure 7 shows that the monoclonal antibodies P113-7D6, P112-4C1, P113-12A6, P112-11D2, P112-10D4, LM609, 23C6 inhibit the proliferation of human dermal microvascular endothelial cells plated on osteopontin. The control  
10 monoclonal antibody, M399, has no inhibitory activity.

Figure 8 shows immunolocalization of  $\alpha_v\beta_3$  in human placenta tissue and human colon carcinoma tissue with monoclonal anti- $\alpha_v\beta_3$  P112-4C1. Panels A and B are  
15 photomicrographs of a section of human placenta that had been frozen, sectioned and stained for detection of the  $\alpha_v\beta_3$  integrin. Panel A shows that staining of the placental tissue using an irrelevant isotype-matched antibody control fails to detect the presence of  $\alpha_v\beta_3$ .  
20 Panel B shows that staining the placental tissue using the monoclonal anti- $\alpha_v\beta_3$  P112-4C1 antibody detects trophoblasts (arrows) and syncytiotrophoblasts (arrowhead) that are positively stained for the presence of the  $\alpha_v\beta_3$  integrin. Panels C and D are  
25 photomicrographs of portions of a section of formalin-fixed, paraffin-embedded human clonic carcinoma that had been processed, sectioned and stained for detection of the  $\alpha_v\beta_3$  integrin. Panel C shows that staining of the human colon carcinoma tissue using an irrelevant  
30 isotype matched antibody control fails to detect the presence of  $\alpha_v\beta_3$ . Panel D shows that the monoclonal anti- $\alpha_v\beta_3$  P112-4C1 antibody detects positive staining in neoplastic enterocytes (arrow), endothelial cells (arrowhead) and fibroblasts (open arrowhead) in human  
35 colon carcinoma tissue.



## DETAILED DESCRIPTION OF THE INVENTION

## Metastases

5 Integrins are believed to be involved in, amongst other biological processes, tumor metastasis. Studies have shown, (Humphries, et al., J. Clin. Invest., Vol. 81 (1988) 782), that RGD-like compounds can interfere with experimental metastasis wherein tumor cells are  
10 injected directly into the blood.

Tumor cell invasion occurs by a three step process:

- 1) tumor cell attachment to extracellular matrix;
  - 2) proteolytic dissolution of the matrix; and
  - 15 3) movement of the cells through the dissolved barrier.
- This process can occur repeatedly and can result in metastases at sites distant from the original tumor.

Seftor et al. (Proc Natl. Acad. Sci. USA, Vol. 89  
20 (1992) 1557) have shown that the  $\alpha_v\beta_3$  integrin has a biological function in melanoma cell invasion. Montgomery et al., (Proc. Natl. Acad. Sci. USA, Vol. 91 (1994) 8856) have demonstrated that the integrin  $\alpha_v\beta_3$  expressed on human melanoma cells promotes a survival  
25 signal, protecting the cells from apoptosis. Accordingly, mediation of the tumor cell metastatic pathway by interference with the  $\alpha_v\beta_3$  integrin cell adhesion receptor to impede tumor metastasis would be beneficial.

30 Brooks et al. (Cell, Vol. 79 (1994) 1157) have demonstrated that antagonists of  $\alpha_v\beta_3$  provide a therapeutic approach for the treatment of neoplasia (inhibition of solid tumor growth) since systemic  
35 administration of  $\alpha_v\beta_3$  antagonists causes dramatic regression of various histologically distinct human tumors.



The monoclonal antibodies of the present invention may also be used in a method of treating  $\alpha_v\beta_3$  integrin-mediated diseases, such as tumor metastasis by administering to a mammal in need of treatment an effective amount of an  $\alpha_v\beta_3$  integrin antibody that acts as an  $\alpha_v\beta_3$  integrin antagonist or inhibitor. Reference is made to co-pending application number (docket no. 2046.76), contemporaneously filed herewith in the names of Christopher P. Carron, Debra M. Meyer, G. Allan Nickols and Jeffrey W. Smith and entitled Methods For Using Anti- $\alpha_v\beta_3$  Integrin Antibody Antagonists, the disclosure of which is incorporated by reference herein.

15

#### Tumor Growth/Angiogenesis

The adhesion receptor integrin  $\alpha_v\beta_3$  was identified as a marker of angiogenic blood vessels in chick and man and plays a critical role in angiogenesis or neovascularization (Brooks et al., J-Clin-Invest, Vol. 96 (1995) 1815). Angiogenesis is characterized by the invasion, migration and proliferation of smooth muscle and endothelial cells. Antagonists of  $\alpha_v\beta_3$  inhibit this process by selectively promoting apoptosis of cells in neovasculature. The growth of new blood vessels, or angiogenesis, also contributes to pathological conditions such as diabetic retinopathy (Adonis et al., Amer. J. Ophthal., Vol. 118, (1994) 445), rheumatoid arthritis (Peacock et al., J. Exp. Med., Vol. 175, (1992) 1135) and osteoarthritis (Ondrick et al., Clin.-Podiatr.-Med.-Surg., Vol. 9, (1992) 185). Therefore,  $\alpha_v\beta_3$  antagonists would be useful therapeutic targets for treating such conditions associated with neovascularization (Brooks et al., Science, Vol. 264, (1994) 569). Because angiogenesis occurs normally in the female reproductive organs, antagonists of  $\alpha_v\beta_3$  would be useful in controlling fertility.

### Osteoporosis

It has been reported that the cell surface receptor  $\alpha_v\beta_3$  is the major integrin on osteoclasts responsible for attachment to bone. Osteoclasts cause bone resorption and when such bone resorbing activity exceeds bone forming activity it results in osteoporosis (a loss of bone), which leads to an increased number of bone fractures, incapacitation and increased mortality.

5 Antagonists of  $\alpha_v\beta_3$  have been shown to be potent inhibitors of osteoclastic activity both *in vitro* (Sato et al., J. Cell. Biol., Vol. 111 (1990) 1713) and *in vivo* (Fisher et al., Endocrinology, Vol. 132 (1993) 1411. Antagonism of  $\alpha_v\beta_3$  leads to decreased bone

15 resorption and therefore restores a normal balance of bone forming and resorbing activity. Thus it would be beneficial to provide antagonists of osteoclast  $\alpha_v\beta_3$ , which are effective inhibitors of bone resorption and therefore are useful in the treatment or prevention of

20 osteoporosis.

### Restenosis

The role of the  $\alpha_v\beta_3$  integrin in smooth muscle cell migration also makes it a therapeutic target for

25 prevention or inhibition of neointimal hyperplasia which is a leading cause of restenosis after vascular procedures (Choi et al., J. Vasc. Surg. Vol. 19(1) (1994) 125). Prevention or inhibition of neointimal

30 hyperplasia by pharmaceutical agents to prevent or inhibit restenosis would be beneficial.

### Viral Infection

35 White (Current Biology, Vol. 3(9) (1993) 596) has reported that adenovirus uses  $\alpha_v\beta_3$  for entering host cells. The integrin appears to be required for endocytosis of the virus particle and may be required

for penetration of the viral genome into the host cell. Thus antibodies which inhibit  $\alpha_v\beta_3$  would find usefulness as antiviral agents.

## 5 Anti-Integrin Antibodies

A number of anti-integrin antibodies are known. Doerr et al. (J.B.C., Vol. 271 (1996) 2443) reported that a blocking antibody to  $\alpha_v\beta_3$  integrin in vitro inhibits the  
10 migration of MCF-7 human breast cancer cells in response to stimulation from IGF-1. Gui et al. (British J. Surgery, Vol. 82 (1995) 1192) report that antibodies against  $\alpha_v\beta_3$  and  $\beta_1$  and  $\beta_3$  inhibit in vitro chemoinvasion by human breast cancer carcinoma cell  
15 lines Hs578T and MDA-MB-231. Lehman et al. (Cancer Research, Vol. 54 (1994) 2102) show that a monoclonal antibody (69-6-5) reacts with several  $\alpha_v$  integrins including  $\alpha_v\beta_3$  and inhibited colon carcinoma cell adhesion to a number of substrates, including  
20 vitronectin. Brooks et al. (Science, Vol. 264 (1994) 569) show that blockade of integrin activity with an anti- $\alpha_v\beta_3$  monoclonal antibody inhibits tumor-induced angiogenesis of chick chorioallantoic membranes by human M21-L melanoma fragments. Chuntharapai et al.  
25 (Exp. Cell. Res., Vol. 205 (1993) 345) discloses monoclonal antibodies 9G2.1.3 and 10C4.1.3 which recognize the  $\alpha_v\beta_3$  complex, the latter monoclonal antibody is said to bind weakly or not at all to tissues expressing  $\alpha_v\beta_3$  with the exception of  
30 osteoclasts and was suggested to be useful for in vivo therapy of bone disease. The former monoclonal antibody is suggested to have potential as a therapeutic agent in some cancers.

35 Ginsberg et al., U.S. Patent No. 5,306,620 discloses antibodies that react with integrin so that the binding affinity of integrin for ligands is increased. As such these monoclonal antibodies are said to be useful for

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preventing metastasis by immobilizing melanoma tumors. Brown, U.S. Patent No. 5,057,604 discloses the use of monoclonal antibodies to  $\alpha_v\beta_3$  integrins that inhibit RGD-mediated phagocytosis enhancement by binding to a  
5 receptor that recognizes RGD sequence containing proteins. Plow et al., U.S. Patent No. 5,149,780 discloses a protein homologous to the RGD epitope of integrin  $\beta$  subunits and a monoclonal antibody that inhibits integrin-ligand binding by binding to the  $\beta_3$   
10 subunit. That action is said to be of use in therapies for adhesion-initiated human responses such as coagulation and some inflammatory responses.

As a result of the present invention, monoclonal  
15 antibodies can be used in a method for blocking  $\alpha_v\beta_3$ -mediated events such as cell adhesion, osteoclast-mediated bone resorption, restenosis, ocular neovascularization and growth of hemangiomas, as well as neoplastic cell or tumor growth and dissemination.  
20 The inventive monoclonal antibodies can also be used for antibody-mediated targeting and delivery of therapeutics for disrupting or killing  $\alpha_v\beta_3$  bearing neoplasms and tumor-related vascular beds. In addition, the inventive monoclonal antibodies can be  
25 used for visualization or imaging of  $\alpha_v\beta_3$  bearing neoplasms or tumor related vascular beds, for example, by NMR or immunoscintigraphy.

In addition, these monoclonal antibodies detect  $\alpha_v\beta_3$  in  
30 solution and in frozen tissue sections and on the surface of cells and therefore these monoclonal antibodies may be used for the detection and characterization of  $\alpha_v\beta_3$ -bearing tumor and endothelial cells in human malignancies. Accordingly, the  
35 monoclonal antibodies of the present invention may be used for immunochemical and immunohistochemical identification of tumor vasculature. Since the integrin  $\alpha_v\beta_3$  is minimally expressed on resting or

normal blood vessels, but is significantly up-regulated on vascular cells within human tumors (Brooks et al., Cell, Vol. 79 (1994) 1157; Brooks et al., Science, Vol. 264 (1994) 569; Brooks, et al., J-Clin-Invest, Vol. 96 (1995) 1815),  $\alpha_v\beta_3$  may be considered a marker of human tumor-associated blood vessels and tumor growth (Brooks, et al., J-Clin-Invest, Vol. 96 (1995) 1815). Consequently, antibodies that recognize  $\alpha_v\beta_3$  may be used as an immunodiagnostic agent to identify tumor-related blood vessels or vascular beds using conventional immunohistochemical techniques. Further, since the integrin  $\alpha_v\beta_3$  is a marker of tumor-associated blood vessels and recent findings indicate that blood vessel density is a prognostic indicator of cancer and disease status (Weidner, Semin-Diagn-Pathol, Vol. 10 (1993) 302) antibody that recognize  $\alpha_v\beta_3$  may be used as a component of a diagnostic device or technique to measure tumor-related blood vessel abundance or density and to define disease prognosis. Specifically, histologic sections from fresh-frozen or formalin-fixed paraffin embedded tumor tissue are immunostained with  $\alpha_v\beta_3$  antibody, preferably using the  $\alpha_v\beta_3$  antibody at concentration of 0.1-50  $\mu\text{g/ml}$ , using techniques and procedures known to those skilled in the art.  $\alpha_v\beta_3$  antibody bound to vascular endothelial cells in the tissue can be visualized using a secondary agent, usually a second antibody, that permits localization of the  $\alpha_v\beta_3$  antibody binding within the tissue section. Preferably the second antibody reacts or binds with the  $\alpha_v\beta_3$  antibody and has an attached reporter molecule, e.g., an anti-mouse antibody with an attached enzyme or fluorescent marker. The number and area of immunostained blood vessels in the tissue can then be counted by microscopic techniques, familiar to those skilled in the art, to assess tumor blood vessel density or abundance.

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The monoclonal antibodies of the present invention can also be used in methods for delivering either cytotoxic or cytostatic compounds, nucleic and deoxynucleic acids or radioisotopes to cells having  $\alpha_v\beta_3$ -integrin. For example, the monoclonal antibodies of the present invention (or a Fab, Fab', F(ab')<sub>2</sub> and F(v) fragments thereof containing a paratope for  $\alpha_v\beta_3$ -integrin) can be conjugated to the cytotoxic or cytostatic compounds, nucleic acids or radioisotopes and then contacted with tissue or cells *in vivo* or *in vitro*.

#### Antibodies and Antibody Compositions

The term "antibody" refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope. Illustrative antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', F(ab')<sub>2</sub> and F(v).

The term "antibody combining site" refers to that structural portion of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) antigen. The term "immunoreact" refers to binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof. The term "antigenic determinant" refers to the actual structural portion of the antigen that is immunologically bound by an antibody combining site. The term is also used interchangeably with "epitope".

As used herein, the term "specifically binds" refers to a non-random binding reaction between two molecules,



for example between an antibody molecule immunoreacting with an antigen or between a cell surface integrin receptor and a ligand molecule. Illustrative of a specifically-bound receptor-ligand complex is that  
5 between platelet  $\alpha_{\text{Ib}}\beta_{\text{IIIa}}$  and fibrinogen at the platelet surface.

The ligand to which an integrin specifically binds is referred to as a specific ligand, and must necessarily  
10 be recited in the context of a particular integrin. Specific ligands for binding to integrins are well characterized for many integrins. For example fibrinogen is a specific ligand for the platelet receptor ( $\alpha_{\text{Ib}}\beta_{\text{IIIa}}$ ); vitronectin, von Willebrand factor and  
15 fibrinogen are specific ligands for the vitronectin receptor (VnR); fibronectin is a specific ligand for the VLA-5 receptor; laminin is a specific ligand for the VLA-6 receptor; and collagen is a specific ligand receptor for the VLA-2 receptor. As indicated in  
20 Figure 1 (panel B), the monoclonal antibodies of the present invention do not bind to  $\alpha_{\text{Ib}}\beta_{\text{IIIa}}$ .

A monoclonal antibody composition is typically composed of antibodies produced by clones of a single cell  
25 called a hybridoma that secretes (produces) one kind of antibody molecule. Historically, the hybridoma cells are formed by fusing an antibody-producing cell and a myeloma or other self-perpetuating cell line. Such antibodies were first described by Kohler and Milstein,  
30 Nature, vol. 256 (1975) 495-497, which description is incorporated by reference, although numerous well known variations have since been described for producing hybridoma cells.

35 The preparation of monoclonal antibodies generally involves immunizing a mammal with an inoculum containing the integrin against which the antibody is to immunoreact, thereby inducing in the mammal antibody



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molecules having the immunospecificity described herein. The antibody-producing cells are then isolated, cloned and screened for the presence of antibody molecules of interest.

5

The integrin inhibiting monoclonal antibodies of this invention may be prepared by the method comprising the steps of:

- 10 (a) Immunizing an animal with an inoculum comprising an integrin. The integrin can be presented in a variety of forms, as described herein including purified integrin, partially isolated integrin in the form of cell membranes having the cell surface integrin  
15 receptor associated with the membranes, and whole cells having the integrin associated with the cell membrane.

The immunization is typically accomplished by administering the inoculum to an immunologically  
20 competent mammal in an immunologically effective amount, i.e., an amount sufficient to produce an immune response. Preferably, the mammal is a rodent such as a rabbit, rat or mouse. The mammal is then maintained for a period sufficient for the mammal to produce cells  
25 secreting antibody molecules that immunoreact with the receptor.

The word "inoculum" is used herein to describe a composition containing an integrin in one of the forms  
30 described above as an active ingredient used for the preparation of integrin-inhibiting antibodies.

The inoculum contains an effective, immunogenic amount of an  $\alpha_v\beta_3$  integrin. The effective amount of integrin  
35 per unit dose sufficient to induce an immune response to the immunizing integrin depends, among other things, on the species of animal inoculated, the body weight of the animal and the chosen inoculation regimen as is

well known in the art. Inocula typically contain protein concentrations of about 10 micrograms to about 500 milligrams per inoculation (dose), preferably about 50 micrograms to about 50 milligrams per dose.

5

The term "unit dose" as it pertains to the inocula refers to physically discrete units suitable as unitary dosages for animal, each unit containing a predetermined quantity of material calculated to produce the desired immunogenic effect in association with the required diluent; i.e., carrier or vehicle. The specifications for the novel unit dose of an inoculum of this invention are dictated by and are directly dependent on (a) the unique characteristics of the active material and the particular immunologic effect to be achieved, and (b) the limitations inherent in the art of compounding such active material for immunologic use in animals, as disclosed in detail herein, these being features of the present invention.

15  
20

Inocula are typically prepared from the immunogen, for example an isolated integrin, by dispersing the immunogen in a physiologically tolerable diluent such as water, saline or phosphate-buffered saline to form an aqueous composition.

Inocula can also include an adjuvant as part of the diluent. Adjuvants such as Freund's Complete Adjuvant (FCA), Freund's Incomplete Adjuvant (FIA) and alum are material well known in the art, and are available commercially from several sources.

In a preferred immunization, mice were immunized 3 times over a period of several weeks with  $1 \times 10^6$  baby hamster kidney (BHK) cells that had been engineered to produce  $\alpha_v\beta_3$ . BHK cells were transfected using LipofectAMINE, according to the manufacturer's recommendations (Gibco BRL, Gaithersburg, MD), with

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human  $\alpha_v$  or  $\beta_3$  cDNA in the mammalian expression vector pcDNA3 (Invitrogen Corporation) which confers Neomycin resistance (Southern, P.J. and Berg, P., J. Mol. Appl. Gen., Vol. 1 (1982) 327). Expression of human  $\alpha_v\beta_3$  by  
5 transfected BHK cells was confirmed by flow cytometry using the  $\alpha_v\beta_3$ -specific mAb LM609 (Chemicon, Temecula, CA; Proc. Natl. Acad. Sci. USA, Vol. 84 (1987) 6471. Mice were immunized with  $\alpha_v\beta_3$ -expressing BHK cells emulsified in FCA at the time of the first  
10 immunization; all other immunizations utilized antigen mixed with FIA or phosphate buffered saline. Approximately four days prior to harvesting splenocytes from mice immunized with cells, the mice were immunized with  $\alpha_v\beta_3$  purified from human placental extracts.  
15

(b) A suspension of antibody-producing cells removed from the immunized mammal is then prepared. This is typically accomplished by removing the spleen of the mammal and mechanically separating the individual  
20 spleen cells in a physiologically tolerable medium using methods well known in the art.

(c) The suspended antibody-producing cells are treated with a transforming agent capable of producing a  
25 transformed ("immortalized") cell line. Transforming agents and their use to produce immortalized cell (transformed) lines are well known in the art and include DNA viruses such as Epstein Bar Virus (EBV), Simian Virus 40 (SV40), Polyoma Virus and the like, RNA  
30 viruses such as Moloney Murine Leukemia Virus (Mo-MuLV), Rous Sarcoma Virus and the like, myeloma cells such as P3X63-Ag8.653, Sp2/0-Ag14 and the like. In preferred embodiments, treatment with the transforming agent results in the production of a  
35 hybridoma by means of fusing the suspended spleen cells with mouse myeloma cells from a suitable cell line by the use of a suitable fusion promoter. A preferred ratio is about 2 spleen cells per myeloma cell in a

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suspension containing about  $10^8$  splenocytes. A preferred fusion promoter is polyethylene glycol having an average molecule weight from about 1000 to about 4000 (commercially available as PEG 1300-1600, ATCC);  
5 however, other fusion promoters known in the art maybe employed.

The cell line used should preferably be of the so-called "drug resistant" type, so that unfused  
10 myeloma cells will not survive in a selective medium, while hybrids will survive. A common class is 8-azaguanine resistant cell lines, which lack the enzyme hypoxanthine-guanine phosphoribosyl transferase and hence will not be supported by HAT (hypoxanthine,  
15 aminopterin, and thymidine) medium. It is also generally preferred that the myeloma cell line used be of the so-called "non-secreting" type which does not itself produce any antibody. In certain cases, however, secreting myeloma lines may be preferred.

20

(d) The transformed cells are then cloned, preferably to monoclonality. The cloning is preferably performed in a tissue culture medium that will not sustain (support) non-transformed cells. When the transformed  
25 cells are hybridomas, this is typically performed by diluting and culturing in separate containers the mixture of unfused spleen cells, unfused myeloma cells, and fused cells (hybridomas) in a selective medium which will not sustain the unfused myeloma cells  
30 (non-transformed cells). The cells are cultured in this medium for a time sufficient to allow death of the unfused cells (about one week). The dilution can be a limiting dilution, in which the volume of diluent is statistically calculated to isolate a certain number of  
35 cells (e.g., 1-4) in each separate container (e.g., each well of a microtiter plate). Alternatively, the dilution may be done in soft agar such that a single cell suspension is generated and then plated in

semisolid agar (Goding, J.W., J. Immunol. Methods., Vol. 39 (1980) 285). The medium is one (e.g., HAT medium) that will not sustain the drug-resistant (e.g., 8-azaguanine resistant) unfused myeloma cell line.

5

(e) The tissue culture medium of the cloned hybridomas is then assayed to detect the presence of secreted antibody molecules having the immunoreactive properties as described herein using well known immunological screening techniques together with the assays described herein to identify integrin-inhibiting antibodies.

As shown by the various screening protocols in Example 1, below, to identify integrin-inhibiting antibodies, several separate assays are typically conducted to identify an antibody of this invention. First, the culture is evaluated for antibodies immunoreactive with  $\alpha_v\beta_3$ . Hybridoma in cell cultures containing the  $\alpha_v\beta_3$ -reactive antibodies are then cloned by dilution and individual clones re-screened to identify clonal cell lines producing  $\alpha_v\beta_3$ -reactive antibodies.

Representative and preferred methods for producing integrin-inhibiting monoclonal antibody compositions are described in Example 1, below.

(f) Desired clonal cell lines are then selected. To produce a much greater concentration of monoclonal antibody, the desired hybridoma can be transferred by injection into mice, preferably syngeneic or semisyngeneic mice. The hybridoma will cause formation of antibody-producing tumors after a suitable incubation time, which will result in a high concentration of the desired antibody (about 5-20 mg/ml) in the bloodstream and peritoneal exudate (ascites) of the host mouse.

A monoclonal antibody composition can be enriched in the desired antibody molecules by additional isolation methods such as immunoaffinity chromatography using solid phase affixed immunizing antigens, as described  
5 herein, or by using, for example, DEAE Sephadex to obtain the IgG fraction, if desired.

Media and animals useful for the preparation of these compositions are both well known in the art and  
10 commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DME/F12, 1:1) supplemented with 4.5 gm/l glucose, 20 mM glutamine, and 20% fetal calf serum. An exemplary  
15 inbred mouse strain is the Balb/c.

A monoclonal antibody composition can also be produced by methods well known to those skilled in the art of producing antibodies by recombinant DNA methods. Those  
20 methods include isolating, manipulating, and expressing the nucleic acid that codes for all or part of an immunoglobulin variable region including both the portion of the variable region comprised by the variable region of immunoglobulin light chain and the  
25 portion of the variable region comprised by the variable region of immunoglobulin heavy chain. Methods for isolating, manipulating, and expressing the variable region coding nucleic acid in procaryotic and eucaryotic hosts are disclosed in Robinson et al., PCT  
30 Publication No. WO 89/0099; Winter et al., European Patent Publication No. 0239400; Reading, U.S. Pat. No. 4,714,681; Cabilly et al., European Patent Publication No. 0125023; Sorge et al., Mol. Cell Biol., Vol. 4 (1984) 1730-1737; Beher et al., Science, Vol. 240  
35 (1988) 1041-1043; Skerra et al., Science, Vol. 240 (1988) 1030-1041; and Orlandi et al., Proc. Natl. Acad. Sci., U.S.A., Vol. 86 (1989) 3833-3837. Typically the nucleic acid codes for all or part of an immunoglobulin



- variable region that binds a preselected antigen (ligand). Sources of such nucleic acid are well known to one skilled in the art and, for example, may be obtained from a hybridoma producing a monoclonal
- 5 antibody that binds the preselected antigen, or the preselected antigen may be used to screen an expression library coding for a plurality of immunoglobulin variable regions, thus isolating the nucleic acid.
- 10 See, for example, the method of isolating monoclonal antibodies from an immunological repertoire as described by Sastry et al., (Proc. Natl. Acad. Sci., Vol. 86 (1989) 5728-5732); and Huse et al., (Science, Vol. 246 (1989) 1275-1281).
- 15 Isolated nucleic acid coding for the murine immunoglobulin genes may be engineered to prepare  $\alpha_v\beta_3$  antibody combining regions grafted into human Ig backbones using the process known as antibody
- 20 humanization (Richmann et al., Nature, Vol. 332 (1988) 323). Further, nucleic acid coding for the murine immunoglobulin genes of  $\alpha_v\beta_3$  antibodies may be engineered to produce recombinant bifunctional antibodies, single chain Fv (scFv) or bispecific scFv
- 25 fusion proteins that, in a single gene or gene product, combine a toxin, immunostimulatory molecule or targeting moiety with the  $\alpha_v\beta_3$ -binding domain (Reisfeld, et al., Curr Top Microbiol Immunol, Vol. 213 (1996) 27; Rybak et al., Proc. Natl. Acad. Sci. USA, Vol. 89
- 30 (1992) 3165; Siegall et al., J. Immunol., Vol. 152 (1994) 2377; Naramura, et al., Immunol. Lett., Vol. 39 (1993) 91). Further, nucleic acid coding for the murine immunoglobulin genes of  $\alpha_v\beta_3$  antibodies or the nucleic acid coding the humanized engrafted counterpart
- 35 may be engineered to increase antigen-binding affinity using techniques known to those skilled in the art (Rheinnecker et al., J. Immunol., Vol. 157 (1996) 2989; Barbas et al., TBTECH, Vol. 14 (1996) 230; Hoogenboom,



- Trends Biotechnol. Vol. 15(2) (1997) 62). Recombinant  $\alpha_v\beta_3$  antibodies, antibody fragments or fusion proteins derived from the same may be expressed in E coli, transgenic plants or animals (Huse et al., Science, Vol. 246 (1989) 1275; Hiatt et al., Nature, Vol. 342 (1989) 76; Morcol et al., Ann. N. Y. Acad. Sci., Vol. 721 (1994) 218; Ebert et al., Biotechnology-N-Y, Vol. 9 (1991) 835).
- 10 The anti- $\alpha_v\beta_3$  monoclonal antibodies of the present invention may be made by recombinant DNA methods, such as those described in U.S. Patent 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional
- 15 procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be
- 20 inserted in to an expression vector, and then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the
- 25 recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous

30 murine sequences (see Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855, U.S. 4,816,567 and U.S. 5,693,761, which are incorporated by reference herein). Recombinant DNA technology has been used to produce immunoglobulin molecules that have human framework

35 regions combined with complementarity determining regions (CDR's) from a donor mouse or rat immunoglobulin. These new proteins are called "reshaped" or "humanized" immunoglobulins and the

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process by which the donor immunoglobulin is converted into a human-like immunoglobulin by combining its CDR's with a human framework is called "humanization".

Humanized antibodies are important because they bind to  
5 the same antigen as the original antibodies, but are less immunogenic when injected into a human host.

The monoclonal antibody compositions produced by the above method can be used, for example, in diagnostic  
10 and therapeutic modalities wherein specific binding or inhibition of integrin is desired, as described further herein.

(g) IgG isolated from ascites fluids of mice inoculated  
15 with the cell lines producing  $\alpha_v\beta_3$ -reactive antibodies are then further evaluated as inhibitors of  $\alpha_v\beta_3$  in assays such as inhibition of Mn-induced binding of M21 cells to fibrinogen (Figure 2); inhibition of M21 cell migration (Figure 3); inhibition of 293/ $\beta_3$ , 293/ $\beta_5$  or  
20 293/ $\beta_1$  cells to vitronectin (Figures 4 and 5); and inhibition of rabbit osteoclast adhesion to osteopontin (Figure 6).

#### Hybridomas

25 Hybridomas of the present invention are those which are characterized as having the capacity to produce an integrin-inhibiting monoclonal antibody composition of this invention.

30 A preferred hybridoma of the present invention is characterized as producing integrin-inhibiting antibody molecules that immunoreact with an integrin. The antibody also is "complex-specific" in the sense that  
35 it immunoreacts with an integrin  $\alpha_v\beta_3$  complex and does not react with either of the integrin  $\alpha_v$  or  $\beta_3$  subunits individually. In other words, the antibody recognizes  $\alpha_v$  in the context of  $\beta_3$ , while at the same time

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recognizing  $\beta_3$  in the context of  $\alpha_v$ , as shown in Figure 5.

Representative preferred hybridomas are prepared and  
5 described in Example 1, below. Particularly preferred are the hybridoma cell lines designated P113-7D6, P112-4C1, P113-12A6, P112-11D2, P112-10D4 and P113-1F3.

Hybridoma cell lines P113-7D6, P112-4C1, P113-12A6,  
10 P112-11D2, P112-10D4 and P113-1F3 have been deposited pursuant to Budapest Treaty requirements with the American Type Culture Collection (ATCC), Rockville, Me. 20852, U.S.A., on November 5, 1996, and were assigned, respectively, accession numbers HB-12224, HB-12225, HB-  
15 12226, HB-12227, HB-12228 and HB-12229.

These hybridomas were deposited in a depository affording permanence of the deposit and ready accessibility thereto by the public upon the grant of a  
20 patent, under conditions which assure that access to the hybridoma will be available during the pending of the patent application to those entitled to such access, and that all restrictions on the availability to the public of the hybridoma as deposited will be  
25 irrevocably removed upon the granting of the patent. The deposited hybridomas will be maintained by the ATCC, and all maintenance fees have been paid, for the term of the patent or 30 years from the date of deposit, whichever is longer, and in all events for at  
30 least five years after the date of the last request for access.

Methods for producing hybridomas producing (secreting) antibody-molecules having a desired immunospecificity,  
35 i.e., having the ability to immunoreact with a particular antigen, an identifiable epitope on a particular antigen, are well known in the art and are described further herein. Particularly applicable is

the hybridoma technology described by Niman et al.,  
Proc. Natl. Acad. Sci. USA, Vol. 80 (1983)  
4949-4953, and by Galfre et al., Meth. Enzymol., Vol.  
73 (1981) 3-46, which descriptions are incorporated  
5 herein by reference.

#### Therapeutic Methods and Compositions

Therapeutic methods and compositions are contemplated  
10 for inhibiting integrins. These methods are useful to  
inhibit cell attachment and migration mediated by the  
integrins and find application in a wide variety of  
cell types, tissues and systems where attachment of  
cells is desired.

15

Thus in general the invention contemplates a method for  
inhibiting an integrin that binds a specific ligand  
comprising contacting the integrin with a solution  
containing an inhibiting amount of an  
20 integrin-inhibiting antibody as described herein that  
is immunospecific for the integrin, for example  $\alpha_v\beta_3$   
integrin.

Typically the method is practiced on cells expressing  
25 the integrin on the surface of the cell, so the  
contacting occurs by admixing the cells in a solution  
with the integrin-inhibiting antibodies to form an  
admixture. The admixture is preferably physiologically  
compatible with cell viability, preferably sterile, and  
30 more preferably compatible with admixture with blood to  
facilitate adding the admixture to the blood.

An inhibiting amount of integrin-inhibiting antibody is  
an amount sufficient to produce the desired result,  
35 namely to inhibit the integrin to a degree sufficient  
to reduce the adhesion of the cell expressing the  
integrin, and typically depends on the amount of  
integrin to be contacted.

In preferred embodiments, whether the method is practiced in vitro or in vivo, an inhibiting amount is an amount sufficient to provide at least about one molar equivalent of integrin-inhibiting antibody per  
5 molar equivalent of integrin to be inhibited. This amount is referred to as a stoichiometric amount of integrin-inhibiting antibody. Although antibody affinity for immunoreaction is typically sufficient for an integrin-inhibiting antibody to immunoreact  
10 stoichiometrically in dilute solutions, it is preferred that an inhibiting amount is in the range of about 100 nanomolar (nM) to 1 millimolar (mM) preferably in the range of 1 to 100 micromolar ( $\mu$ M) and more preferably about 1 to 10  $\mu$ M.

15 When an integrin-inhibiting method is practiced in vitro, a liquid sample containing integrin, and preferably a physiological fluid containing cells that express cell surface integrin, are admixed with an  
20 inhibiting amount of an integrin-inhibiting antibody of this invention to form a complex. The complex is maintained under biological conditions compatible with the formation of an immunoreaction product and also compatible, if required, with cell viability for a time  
25 period sufficient for the integrin-inhibition antibody to immunoreact with the integrin and, when immunoreacted, inhibit the integrin.

When the integrin-inhibiting method is practiced in  
30 vivo, an inhibiting amount of an antibody composition containing a physiologically tolerable diluent and integrin-inhibiting antibody molecules as described herein is intravenously administered to a mammal, e.g. a human, and the mammal is maintained for a sufficient  
35 period to allow the antibody molecules to immunoreact with any integrin present and form an inhibiting immunoreaction product. Other routes of administration

are envisioned, including intraperitoneal, intramuscular, intrathecal and subcutaneous.

In preferred embodiments, a therapeutic composition for  
5 use in an integrin-inhibiting method includes integrin-inhibiting antibody molecules that immunoreact with the integrin complex, i.e., immunoreact with both the  $\alpha$  and  $\beta$  subunits of the heterodimer of the  
10 subunits. Exemplary compositions comprise one or more of the monoclonal antibodies secreted by the hybridomas P113-7D6, P112-4C1, P113-12A6, P112-11D2, P112-10D4 or P113-1F3.

15 The integrin-inhibiting antibodies may be combined with other pharmaceutical compositions and/or excipients.

For example,  $\alpha_v\beta_3$  antibody may be co-administered or added to established anti-cancer chemotherapeutic or  
20 biotherapeutic regimens. Normal physiologic saline is a preferred excipient or vehicle for administration of antibody. This may include, but is not limited to, combining or co-administering  $\alpha_v\beta_3$  antibodies with cytotoxic drugs, combinations of cytotoxic drugs or  
25 with immune stimulating drugs, such as interleukins or their derivatives or with hematopoietic factors and their derivatives. For example,  $\alpha_v\beta_3$  antibody may be co-administered or added to therapeutic regimens for the use IL-1, IL-2, IL-4, IL-6, IL-12, IL-15, TNF,  $\alpha$ ,  
30  $\beta$ ,  $\gamma$  interferons and M-CSF, or combinations of these agents or their derivatives, in biologic therapy of cancer. Further,  $\alpha_v\beta_3$  antibody may be co-administered or added to therapeutic regimens for the use of G-CSF, M-CSF, IL-3 and erythropoietin, or combinations of  
35 these agents or their derivatives, in biologic therapy of cancer.



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The anti- $\alpha,\beta$ , monoclonal antibodies of the present invention, or fragments thereof containing a paratope (e.g., Fab, Fab', F(ab')<sub>2</sub> and F(v) fragments), may also be combined with cytotoxic or cytostatic drugs, protein  
5 toxins, nucleic acids or radioisotopes by methods well know to those of ordinary skill in the art. Depending on the dose and the nature of the antibody conjugate, it is possible to either kill tumor cells and reduce tumor size or achieve inhibition of cell growth and  
10 hence inhibit tumor growth.

Many monoclonal antibody conjugates of radioisotopes, protein toxins, cytotoxic and cytostatic drugs have been prepared and tested in model systems. Radio-  
15 labelled monoclonal antibody conjugates have proven to be effective imaging agents in the clinic and are showing promising therapeutic results for the treatment of lymphomas and leukemias in humans. Protein toxin monoclonal antibody conjugates are also being tested in  
20 human clinical trials for conditions such as lymphomas. Cytotoxic and cytostatic drugs conjugated to antibodies have also been investigated in clinical trials.

Exemplary procedures for linking cytotoxic and  
25 cytostatic drugs, as well as protein toxins and peptides, to antibodies are described in the CRC Handbook: Chemistry of Protein Conjugation and Cross-linking, Chapter 11, 267 (CRC Press Inc., Boca Raton Fl., 1993), as well as in, for example, U.S. Patent No.  
30 5,591,829; both of which are incorporated by reference herein.

The amino side chains of lysines, the amino terminal group in polypeptides, the carboxyl groups of aspartic  
35 and glutamic acids, the thiol group of cysteins, and the carbohydrate moiety of antibodies, toxins and other drugs have been used to prepare drug conjugates. The CRC Handbook: Chemistry of Protein Conjugation and



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Cross-linking describes, among other things, various heterobifunctional reagents for linking various substances to antibodies. Such reagents include m-maleimido-benzoyl-N-hydroxysuccinimide ester (MBS) and  
5 N succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Disulfide generating agents, such as 3-(2-dipyridyldithio)propionate can be used to generate pyridyl disulfide groups in proteins in conjunction with water soluble carbodiimides such as ethyl-3-(3-  
10 dimethylaminopropyl carbodiimide. Conjugation through thioether linkages can be effected using iodoacetyl compounds, such as N-hydroxysuccinimidyl iodoacetate or N-succinimidyl(4-iodoacetyl)aminobenzoate, while conjugation can also be achieved using acid-labile and  
15 photocleavable cross-linkers, such as 4(iodoacetylamino)-3,4,5,6-tetrahydrophthalic anhydride and 1-[4-nitro-3-(1-chlorocarbonyloxyethyl)phenyl]methyl-S-acetylthioic acid ester. Coupling can also be effected through  
20 carbohydrate residues or linkers, such as dextrans, as well as with polyamino acids, such as poly-L-glutamate.

Various protein toxins, plant toxins, peptides and cytotoxic and cytostatic compounds for conjugation to  
25 antibodies include: ricin, abrin, modeccin, gelonin, pokeweed antiviral protein (PAP),  $\alpha$ -amanitin, ribosome inhibiting protein (RIP), barley RIP, wheat RIP, corn RIP, flax RIP, bacterial toxins including diphtheria toxin, fragments of diphtheria toxin, pseudomonas  
30 exotoxin, shiga toxin and chemical toxins including nitrogen mustard, taxol, doxorubicin, daunomycin, methotrexate, cisplatin, bleomycin, vinblastine, mitomycin C, idarubicin, morpholinodoxorubicin, melphalan, cytosine arabinoside, 5-fluorouracil, and  
35 neocarzinostatin.

For example, SPDP could be used to link free amino groups of antibodies or fragments thereof containing a

paratope (such as Fab, Fab', F(ab')<sub>2</sub> and F(v) fragments) to a variety of plant toxins including ricin, abrin, modeccin, gelonin, pokeweed antiviral protein (PAP),  $\alpha$ -amanitin, ribosome inhibiting protein (RIP), barley RIP, wheat RIP, corn RIP, flax RIP, 5 bacterial toxins including diphtheria toxin, fragments of diphtheria toxin, pseudomonas exotoxin, shiga toxin and chemical toxins including methotrexate, nitrogen mustard, doxorubicin and daunomycin.

10

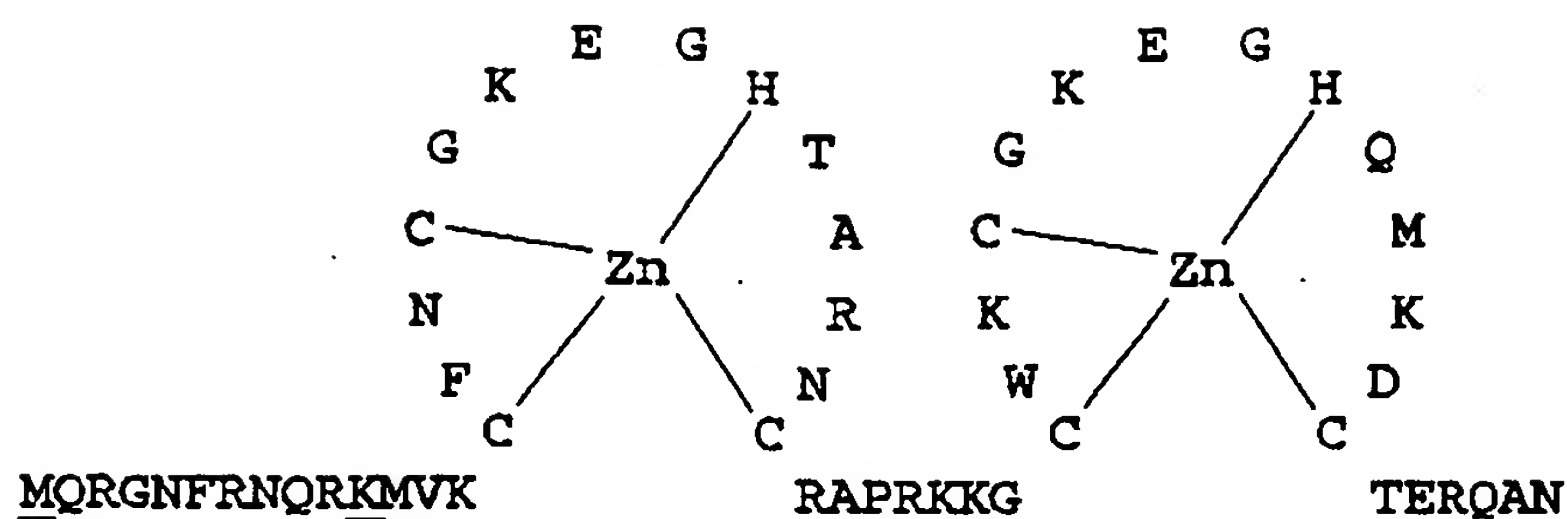
One of ordinary skill in the art can readily prepare antibody conjugates of the following compounds using the antibodies of the present invention: mitomycin C, doxorubicin and daunomycin (Suzuki et al., Chem. Pharm. 15 Bull., Vol. 29 (1981) 844, incorporated by reference herein); idarubicin (Pietersz et al., Cancer Res., Vol. 48 (1988) 926, incorporated by reference herein); doxorubicin (Trail et al., Cancer Res., Vol. 52 (1992) 5693; Trail et al., Science, Vol. 261 (1993) 212; 20 Aboud-Pirak et al., Proc. Natl. Acad. Sci. USA, Vol. 86 (1989) 3778, incorporated by reference herein); morpholinodoxorubicin (Mueller et al., Bioconjugate Chem., Vol. 1, (1990) 325; Dillman et al., Cancer Res., Vol. 48 (1988) 6097); daunomycin (Hurwitz et al., 25 Cancer Res. Vol. 35 (1975) 1175; Page et al., Proc. Am. Assoc. Cancer Res., Vol. 22 (1981) 211; Page et al., "Drug targeting with monoclonal antibodies, in Protides of the Biological Fluids, Peeters, H., Ed., Vol. 29, Pergamon Press, Oxford, (1981) 933; Tsukada et al., J. 30 Natl. Cancer Inst., Vol. 73 (1984) 721; Suzuki et al., Chem. Pharm. Bull., Vol. 29 (1981) 844; Hurwitz, et al., Eur. J. Cancer, Vol. 14 (1978) 1213; Hurwitz et al., J. Appl. Biochem., Vol. 2, (1980) 25; Lavie et al., Cancer Immunol. Immunother., Vol. 33 (1991) 223; 35 Dillman et al., Cancer Res., Vol. 48 (1988) 6097, incorporated by reference herein); vinblastine (Apelgren et al., Cancer Res. Vol. 50 (1990) 3540; Starling et al., Cancer Res., Vol. 51 (1991) 2965;

- 30 -

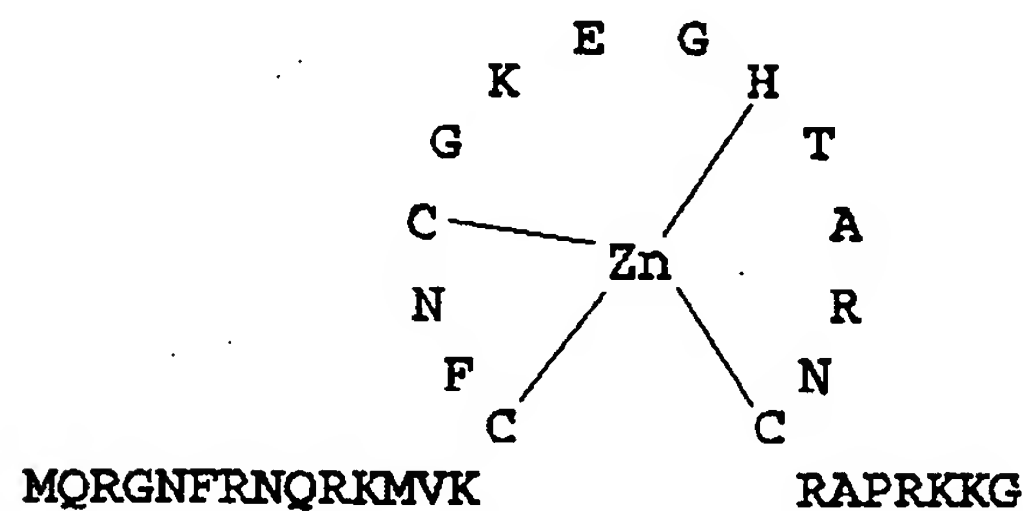
Johnson et al., Cancer Immunol. Immunother., Vol. 27  
(1988) 241, incorporated by reference herein);  
melphalan (Smyth et al., Cancer Res., Vol. 47 (1987)  
62, incorporated by reference herein); cytosine  
5 arabinoside and 5-fluorouracil (Hurwitz et al., J. Med.  
Chem., Vol. 28 (1985) 137, incorporated by reference  
herein); bleomycin (Manabe et al., Biochem. Biophys.  
Res. Commun., Vol. 115 (1983) 1009, incorporated by  
reference herein); neocarzinostatin (Kimura et al.,  
10 Cancer Immunol. Immunother., Vol. 7, (1980) 235,  
incorporated by reference herein); cisplatin (McIntosh  
et al., J. Pharm. Sci., Vol. 86(12) (1997) 1478,  
incorporated by reference herein); methotrexate  
(Kulkarni et al., Cancer Res., Vol. 41 (1981) 2700,  
15 incorporated by reference herein).

Recently, various types of oligodeoxynucleotides  
("ODNs") have demonstrated a broad array of activities  
against distinct intracellular targets, such as  
20 oncogenes, normal host genes, and viral targets.  
Uhlmann et al., Chem. Rev., Vol. 90 (1990) 543; Crooke,  
Biotechnology, Vol. 10 (1992) 882, incorporated by  
reference herein. Chemically modified nuclease  
resistant analogs of ODNs have been shown to display  
25 activity at relatively high concentrations (greater  
than 1 $\mu$ M), whereas unmodified phosphodiester ODNs have  
activities at typically greater than 10  $\mu$ M.  
Complexation of ODNs to anti- $\alpha_v\beta_3$  integrin antibodies  
offers an approach to facilitate delivery of ODNs to  
30 targets sites, such as mammalian (e.g. human) cells  
expressing  $\alpha_v\beta_3$  integrin. Such compositions may be made  
by linking polypeptide sequences which bind ODNs, such  
as the nucleocapsid 7 of HIV-1 or nucleocapsid-derived  
peptides, for example:

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or



to the antibody. Suitable nucleocapsid 7 peptides, as  
 5 well as suitable oligonucleotides (including anti-sense  
 oligonucleotides such as 5'-GTGCCGGGGTCTTCGGGC-3' for  
 c-myb specific mRNA), are identified in Bachmann et  
 al., J. Mol. Med., Vol. 76 (1998) 126, which is  
 incorporated by reference herein. The nucleocapsid 7  
 10 peptides can be made by methods well known in the art  
 and linked to antibodies of the present invention as  
 described in the CRC Handbook: Chemistry of Protein  
 Conjugation and Cross-linking, Chapter 11 (CRC Press  
 Inc., Boca Raton Fl.) for linking proteins and peptides  
 15 to antibodies.

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In place of oligonucleotides, it may be desirable to substitute anti-sense single stranded DNA of a gene, such as the HIV-or herpes simplex virus thymidine kinase (TK) gene, which are described in Wagner et al.,  
5 Proc. Natl. Acad. Sci. USA, Vol. 78 (1981) 1441 and in U.S. Patent No. 5,631,236, incorporated by reference herein. Delivery of anti-sense DNA in such a case may be beneficial in the treatment of viral infection, especially against HIV. Alternatively, it may be  
10 advantageous to deliver complete double stranded DNA encoding for a gene, instead of the oligonucleotide. For example, the TK gene may be delivered to cells as part of gene therapy for treating solid tumors bearing  $\alpha_v\beta_3$  Integrin. In such case, it is envisioned that  
15 subsequently a prodrug which is activated by TK, such as ganciclovir, is administered to the individual so as to activate the prodrug.

Recently, antibody-radionuclide compositions have shown  
20 success in diagnostic and therapeutic applications in mammals, and especially in humans. In general, conjugated antibody-radionuclide compositions involve chemically reacting a chelating agent, which can bind the radionuclide, to the antibody. Numerous chelating  
25 agents are known in the art for this purpose and generally have nitrogen and sulfur donor atoms, such as dithiodiaminocarboxylic acids and dithioamidocarboxylic acids (known as  $N_2S_2$  chelating agents) and thiotriaza chelating compounds (known as  $N_3S$  chelating agents).  
30 Suitable radioisotopes and chelating agents, along with methods for linking the same to antibodies, are disclosed in U.S. Patent No. 5,130,118, which is incorporated by reference herein. Alternatively, radio-halogen labelled compounds can be made and  
35 reacted with antibody, as described in U.S. Patents No. 5,679,318 and 5,252,748, which are incorporated by reference herein.

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Exemplary procedures and compounds for making radiolabelled monoclonal antibody conjugates for radionuclides such as  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{153}\text{Sm}$ ,  $^{90}\text{Y}$ ,  $^{105}\text{Rh}$ ,  $^{131}\text{I}$  are disclosed in U.S. Patent No. 5,679,318. Similarly, 5 exemplary procedures and chelating compounds for making radiolabelled monoclonal antibody conjugates for radionuclides of copper, e.g.  $^{64}\text{Cu}$ ,  $^{67}\text{Cu}$ ; technetium, e.g.,  $^{99\text{m}}\text{Tc}$ ; rhenium, e.g.,  $^{186}\text{Re}$  and  $^{188}\text{Re}$ ; lead, e.g.,  $^{203}\text{Pb}$  and  $^{212}\text{Pb}$ ; palladium, e.g.,  $^{103}\text{Pd}$ ,  $^{109}\text{Pd}$ ; bismuth, 10 e.g.,  $^{212}\text{Bi}$  and gold, e.g.,  $^{198}\text{Au}$  are disclosed in U.S. Patent No. 5,681,927, which is incorporated by reference herein. One of ordinary skill in the art will appreciate that other isotopes of iodine can also be employed, for example  $^{125}\text{I}$ , and other isotopes, for 15 example  $^{241}\text{Am}$ ,  $^{192}\text{Ir}$ , and  $^{111}\text{In}$ , see e.g., U.S. 5,130,118, incorporated by reference herein. Procedures for generation of free sulfhydryl groups on antibodies or antibody fragments are also known, see, e.g., U.S. Patent No. 4,659,839, which is incorporated by 20 reference herein. Because some radionuclides have relatively short half-lives, it may be desirable to prepare chelating-agent-conjugated antibody and then, shortly prior to use, react the chelating compound conjugated-antibody with the radionuclide of interest.

25

The Examples which follow are intended as an illustration of certain preferred embodiments of the invention, and no limitation of the invention is implied.

30

#### EXAMPLES

##### Generation of Monoclonal Antibody

Example 1: Production of P112-4C1-A2-C7-A3.

35

In accordance with the present invention there is disclosed a hybridoma cell line, P112-4C1-A2-C7-A3, produced by hybrid cell line, P112-4C1-A2-C7-A3,



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generated from a mouse immunized with human  $\alpha_v\beta_3$ . A sample of this cell line is deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, USA with accession No. HB-12225.

5 This antibody was produced as follows.

Six week old female Balb/c mice were immunized intraperitoneally (*i.p.*) with  $1 \times 10^6$  BHK cells transfected with  $\alpha_v\beta_3$  (BHK/ $\alpha_v\beta_3$ ) in Freund's Complete  
10 Adjuvant (FCA). Three weeks later the mice were given a booster *i.p.* immunization using  $1 \times 10^6$  BHK/ $\alpha_v\beta_3$  cells in Freund's Incomplete Adjuvant (FIA). Two weeks later the mice were given a second *i.p.* booster of  $1 \times 10^6$  BHK/ $\alpha_v\beta_3$  cells in FIA. After an additional 3 weeks 5  $\mu$ g  
15 purified  $\alpha_v\beta_3$  in saline was given intravenously and *i.p.* Fusions were performed 3 days later.

Immunized mice were sacrificed by CO<sub>2</sub> overdose and a splenectomy performed immediately. The spleen cells  
20 from mice were fused with myeloma P3X63 Ag8.653 (ATCC) using polyethylene glycol, MW 1300-1600 (ATCC). The cells were diluted to  $1 \times 10^6$  cells/ml in HAT selection media, plated in 48 well culture dishes at 0.5 ml/well and grown under selective conditions (HAT medium) that  
25 allow only cells resulting from the fusion of a splenocyte with a myeloma cell to proliferate. Five hundred and seventy wells were plated with cells and 225 showed hybridoma growth. Conditioned media of wells with hybridoma growth were screened for the  
30 presence of antibodies that bind  $^{125}$ I-labeled  $\alpha_v\beta_3$  or  $^{125}$ I-labeled  $\alpha_{IIIb}\beta_{IIIa}$  using the capture assay described below. Ten wells, one of which was well C1 in plate 4 and hence the designation P112-4C1, contained cells that produced an antibody that bound  $\alpha_v\beta_3$  but not  $\alpha_{IIIb}\beta_{IIIa}$ .  
35 Cells in well 4C1 were cloned in soft agar in order to obtain colonies of cells derived from a single cell that produces an antibody that absorbs  $^{125}$ I-labeled  $\alpha_v\beta_3$  from solution. Colonies were picked from soft agar,



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grown in appropriate cell culture media and then rescreened in the  $\alpha_v\beta_3$  capture assay. An  $\alpha_v\beta_3$ -binding colony derived from well A2 was identified and designated P112-4C1-A2. This process was repeated two  
5 additional times to arrive at the final clonally derived cell line designated P112-4C1-A2-C7-A3. The monoclonal antibody produced by this cell line is an IgG1,  $\kappa$ .

- 10 IgG was purified from mouse ascites generated with the P112-4C1-A2-C7-A3 cell line by Protein G or Protein A chromatography as described below and the purified P112-4C1-A2-C7-A3 monoclonal antibody (mAb) tested in various assays to further characterize the properties  
15 of the P112-4C1-A2-C7-A3 as an inhibitor of  $\alpha_v\beta_3$ . Representative examples of the results of these type of assays are shown in Figs 1-7.

#### Purification of Monoclonal Antibody

20

- Ascites fluid was produced separately in mice from each of one of the 6 hybridomas secreting  $\alpha_v\beta_3$  complex-specific antibodies and the control M399 hybridoma. The ascites fluid was clarified to remove the lipid and  
25 purified on a HiTrap Protein G column according to the manufacturer's recommendations (Pharmacia Biotech) or a Protein A column (Pharmacia) using the BioRad Protein A MAPS II buffer system. The eluted fractions were dialyzed extensively against PBS and checked for purity  
30 by SDS-PAGE. The purified antibodies were >90% pure as determined by Coomassie blue stain and comigrated with an IgG1  $\kappa$ , MOPC-21 (Sigma). The purified antibodies were then assayed in the  $\alpha_v\beta_3$  capture assay to determine if they retained the ability to bind  $\alpha_v\beta_3$ . All 6  
35 antibodies from fusions P112 and P113 bound  $^{125}\text{I}$ -labeled  $\alpha_v\beta_3$  in a dose dependent fashion whereas the control IgG1  $\kappa$  antibody, M399, did not. See Figure 1.

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Immunolocalization of  $\alpha_v\beta_3$  in Human Placenta Tissue and Human Colon Carcinoma Tissue

- Sections of frozen human placenta were treated with 3%  
5  $H_2O_2$  for 5 minutes to block endogenous peroxidase activity, washed with PBS and then incubated with 5% BSA in phosphate buffered saline (PBS) and processed for immunohistochemical detection of  $\alpha_v\beta_3$ .
- 10 Formalin-fixed human colon carcinoma tissue was obtained from the Northwestern School of Medicine, Department of Pathology and processed using an automated tissue processor. Samples were embedded in Tissueprep paraffin and cut to 4 micron thick tissue  
15 sections using a Reichert-Jung Microtome and dried onto conventional glass slides at 59°C for 30 minutes. Afterwards, the sections were de-paraffinized with xylene and rehydrated through graded alcohol to distilled water. Rehydrated sections were treated with  
20 3%  $H_2O_2$  for 20 minutes, washed with PBS, incubated in PBS containing 2% non-fat dry milk (solution A) for 20 minutes and processed for immunohistochemical detection of  $\alpha_v\beta_3$ .
- 25 Sections of human placenta and human colon carcinoma incubated with anti- $\alpha_v\beta_3$  monoclonal antibody (P112-4C1) diluted in solution A to a concentration of 18  $\mu g/ml$  or with a commercially-available, isotype-matched irrelevant or control immunoglobulin (Zymed) diluted to  
30 18  $\mu g/ml$  in solution A. After a 1 hour incubation, sections were washed with PBS and then incubated for 40 minutes with biotinylated horse anti-mouse IgG (Vector Laboratories) diluted 1:100 in PBS containing normal horse serum and then washed with PBS. Bound antibody  
35 was detected using a chromogenic avidin-biotin conjugate method (Elite ABC and Vector DAB kit; Vector Laboratories) according to the manufacturers recommended procedures. Sections were then rinsed in

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tap water and counter-stained in Lerner's #3 hematoxylin for 20 seconds, rinsed in tap water, placed in 0.05% acid alcohol for 4 seconds, rinsed in tap water for 1 minute, incubated in blueing hematoxylin reagent for 30  
5 seconds and then in tap water for 1 minute. Finally, sections were dehydrated in ethanol, cleared with xylene and cover-slipped.

Images of sections of human placenta and human colon  
10 carcinoma were captured using a Nikon Microphot-FXA microscope and a Sony DKC-5000 Digital camera. Sections were scanned to locate the selected field of interest, after which, the images were captured by digital camera and downloaded into a photo design  
15 program (Adobe Photoshop 4.0, Adobe Image Systems, Inc.). Scale bars were added to indicate magnification, and the images combined and annotated.

#### $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_{IIIa}$ Capture Assay

20 96-well plates (Dynatech, Chantilly, VA) were coated with 0.5  $\mu\text{g}/\text{well}$  goat anti-mouse IgG Fc-specific monoclonal antibody (Sigma, St. Louis, MO) in borate buffered saline, pH 8.2 overnight at 4°C. Assay plates  
25 were emptied and 200  $\mu\text{l}/\text{well}$  of 1% BSA in PBS and 0.05% Tween-20 was added to block unreacted sites in wells. Following a 2 hour incubation at 37°C, the plates were washed 3 times with saline and 0.05% Tween-20 (TS). Sample (mouse sera, hybridoma supernatant or purified  
30 monoclonal antibody) was added (50  $\mu\text{l}/\text{well}$ ) and incubated 90 min at 37°C. The plates were washed 3 times in TS and approximately 100,000 cpm/well of  $^{125}\text{I}$ -labeled  $\alpha_v\beta_3$  or  $^{125}\text{I}$ -labeled  $\alpha_{IIb}\beta_{IIIa}$  in Tris-buffered saline containing 1 mM  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , and  $\text{Mn}^{++}$ , pH 7.4 and 1% BSA  
35 & 50 mM octylglucoside were added and incubated for 2 hours at 37°C. The plates were then washed 3 times with TS and the wells were counted in a gamma counter.

### Osteopontin Purification

Full-length human osteopontin cDNA was expressed in *E. coli* as a 6xHis-fusion protein using the QIAexpress pQE  
5 expression system as described by the manufacturer  
(Qiagen, Chatsworth CA). Osteopontin was purified by  
Ni-NTA affinity chromatography according to the  
manufacturers recommendations (Qiagen) and then further  
purified by MonoQ (Pharmacia, Piscataway, NJ)  
10 chromatography. Osteopontin was judged greater than  
95% pure by SDS gel electrophoresis.

### Purification of the Vitronectin Receptor ( $\alpha_v\beta_3$ )

15 Human vitronectin receptor ( $\alpha_v\beta_3$ ) and the human platelet  
receptor ( $\alpha_{IIb}\beta_{IIIa}$ ) was purified from human placenta as  
previously described (Pytela, R., Pierschbacher, M.D.,  
Argaves, S., Suzuki, S., and Rouslahti, E., Methods in  
Enzymology, Vol. 144 (1987) 475; Yatohgo, T., Izumi,  
20 M., Kashiwagi, H., and Hayashi, M., Cell structure and  
Function, Vol. 13 (1988) 281).  $^{125}\text{I}$ -labeled  $\alpha_v\beta_3$  and  $^{125}\text{I}$ -  
labeled  $\alpha_{IIb}\beta_{IIIa}$  were generated by iodinating the unlabeled  
precursors using Iodogen according to the  
recommendation of the manufacturer.

25

### M21 Migration Assay

M21 Melanoma cell line was obtained from J.W. Smith  
(Burnham Institute, La Jolla) and maintained in RPMI  
30 1640 supplemented with 10% FBS, pen-strep (200 U/ml)  
and L-glut (2 mM). Fibrinogen and vitronectin were  
purified from human plasma as previously described  
(Yatohgo et al., Cell Struct. Func., Vol. 13 (1988)  
281; Fuller, et al., Methods in Enzymology Vol. 163  
35 (1988) 44474).

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Extracellular matrix proteins (eg., fibrinogen) were coated on the bottom of Costar transwell membranes at a concentration of 5  $\mu\text{g/ml}$ . Following a 2 hour coating period at 37°C, the migration assay was initiated by the addition of  $2 \times 10^5$  cells to the upper chamber of the reservoir. Migration assays were performed in HBSS, 50 mM HEPES, 1 mg/ml BSA, 0.5 mM  $\text{Ca}^{2+}$ , 0.5 mM  $\text{Mg}^{2+}$ , and 0.2 mM  $\text{Mn}^{2+}$ . Cells were allowed to migrate for 18 hours or 20 hours at 37°C. The upper reservoir was gently swabbed with a cotton-tipped applicator to remove cells from the upper filter surface. The filter was removed and cells on the lower surface were stained with Diff-Quik (Baxter). Cells occupying three to five random high power microscopic fields were counted and averaged. For inhibition studies, the inhibitors were incubated with the cells for 30 minutes at 37°C before addition to the transwell.

#### M21 Cell Adhesion Assay

Fibrinogen was diluted to 10  $\mu\text{g/ml}$  in coating buffer (20mM Tris·HCl, 150mM NaCl, pH 7.4) and added (100  $\mu\text{l}$ ) per well of Immulon2 96-well plates (Dynatech) and incubated overnight at 4°C. The plates were washed (50mM Tris·HCl, 150mM NaCl, pH 7.4) and then available protein binding sites blocked by addition of adhesion buffer (1 X Hanks Balanced Salt Solution without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Sigma), plus 50mM Hepes, pH 7.4) containing 1% BSA.

M21 cells were washed with HBSS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free), incubated in cell dissociation solution (Sigma) for 5 minutes at 37°C and then washed by centrifugation/resuspension in adhesion buffer containing 200 $\mu\text{M}$   $\text{Mn}^{2+}$ .

Cells and various concentrations of mAb were combined in adhesion buffer containing 200 $\mu\text{M}$   $\text{Mn}^{2+}$ , added to

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plates and incubated for 30 minutes at 37°C. The plates were gently washed with 50mM Tris·HCl, 150mM NaCl, pH 7.4, and 100μL of cell lysis/substrate buffer (50mM Na acetate, pH 5.0 containing 0.5% TritonX-100 and 0.3 mg/ml of *p*-nitrophenyl phosphate (Sigma)) was added to each well and incubated for 1 hour at room temperature; 50μL of 1N NaOH was added to develop color and the plate absorbance was measured at 412nm. The number of cells bound/well were determined by reference to a standard curve generated with known numbers of cells.

#### 293 Cell Binding Assay

Human vitronectin was purified from fresh frozen plasma as previously described (Yatohgo, T., Izumi, M., Kashiwagi, H., and Hayashi, M., Cell structure and Function, Vol. 13 (1988) 281). 293 cells were transfected with the  $\beta_3$  and  $\beta_5$  integrin subunits as described below.

96-well plates (Immunlon2, Dynatech, Chantilly, VA) were coated with vitronectin, 50 μl/well in TS (0 mM Tris, 150 mM NaCl, pH 7.4 (TS), overnight at 4°C. Unreacted sites were blocked with 1% BSA in TS, 200 μl/well for 2 hrs at 37°C. Antibodies were diluted in Hank's Buffered Salt Solution (Ca<sup>+</sup>Mg<sup>+</sup> free) (HBSS) & 20 mM Hepes & 0.1% BSA & 200 μM MnCl. 293 cells transfected with  $\beta_3$  (293/ $\beta_3$ ), 293 cells transfected with  $\beta_5$  (293/ $\beta_5$ ), and untransfected 293 cells (293/ $\beta_1$ ) were removed from flasks with Cell Dissociation Buffer (Sigma, St. Louis, MO) and washed 3 times with HBSS. Cells were counted and diluted to 2X10<sup>6</sup> cells/ml in HBSS & 20 mM Hepes & 0.1% BSA & 200 μM MnCl. Equal volumes of cells were mixed with diluted antibodies and incubated 30 min at 37°C. 100 μl/well of cell/antibody mixture was then added to vitronectin coated plates and plates were incubated for 30 min at 37°C. Plates were



washed 3 times with HBSS to remove unbound cells and 100  $\mu$ l of HBSS & 200  $\mu$ M MnCl was added per well. The MTT kit (Promega) was used to detect the number of viable attached cells.

5

#### Flow Cytometry Methods

2 X 10<sup>5</sup> to 5 X 10<sup>5</sup> cells were incubated with 1  $\mu$ g primary antibody in FACS buffer (1% BSA/PBS + 0.1% NaN<sub>3</sub>) at 4°C for 30-60 min., washed, then incubated 30-45 min. at 4°C with a FITC-conjugated goat anti-mouse F(ab')<sup>2</sup> secondary antibody (Zymed Laboratories, Inc., San Francisco, CA). Cells were washed and resuspended in buffer with propidium iodide (PI), then analyzed by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems; San Jose, CA). Viable cells were gated on the basis of PI exclusion.

Table 1 shows the binding of anti-integrin monoclonal antibodies to 293/wild type (wt), 293/ $\beta_3$  and 293/ $\beta_5$  cells using immunofluorescence and flow cytometry. The anti- $\alpha_v\beta_3$  antibodies P113-7D6, P112-4C1, P113-12A6, P112-11D2, P112-10D4, P113-1F3 and LM609, bind to 293 cells transfected with the human  $\beta_3$  cDNA (293/ $\beta_3$  cells), but not wild type 293 cells (293/wt) or 293 cells transfected with human  $\beta_5$  cDNA (293/ $\beta_5$ ). The complex-specific antibody, P1F6 ( $\alpha_v\beta_5$ ; Chemicon), binds only to 293/ $\beta_5$  cells and P4C10 ( $\beta_1$ ; GIBCO) binds to all three cells lines, consistent with the previous report showing that wild type 293 cells express  $\alpha_v\beta_1$ , but not  $\alpha_v\beta_3$  (Bodary and McLean, J. Biol. Chem. Vol. 265 (1990) 5938).



Table 1

Cell line	293/wt 293/ $\beta_3$ 293/ $\beta_5$		
	293/wt	293/ $\beta_3$	293/ $\beta_5$
P4C10 ( $\beta_1$ )	++	++	++
P1F <sub>6</sub> ( $\alpha_v\beta_5$ )	-	-	++
LM609	-	++	-
P113-7D6	-	++	-
P113-1F3	-	++	-
P112-10D4	-	++	-
P113-12A6	-	++	-
P112-4C1	-	++	-
P112-11D2	-	++	-

Table 1 shows  $\alpha_v\beta_3$  antibody binding to 293/wt, 293/ $\beta_3$ , and 293/ $\beta_5$  cells by immunofluorescence and flow cytometry. Cell staining fluorescence intensity of specific antibodies is reported relative to the control antibody M399: (-) <2-fold; (+/-) 2 to 5-fold; (+) 5 to 10-fold; (++) 10 to 50-fold. The cells lines are designated wild type 293 (293/wt), 293 cells transfected with human  $\beta_3$  (293/ $\beta_3$ ) and 293 cells transfected with human  $\beta_5$  (293/ $\beta_5$ ).

#### 293 Cell Engineering

293 cells naturally express  $\alpha_v\beta_1$ ,  $\alpha_2\beta_1$ ,  $\alpha_5\beta_1$ , and  $\alpha_6\beta_1$  with little or no detectable  $\alpha_v\beta_3$  (Bodary & McLean, J. Biol. Chem., Vol. 265 (1990) 5938). Cells were transfected using LipofectAMINE according to the manufacturer's recommendations (Gibco BRL, Gaithersburg, MD) with  $\beta_3$  or  $\beta_5$  cDNA in the mammalian expression vector pcDNA3 (Invitrogen Corporation) which confers Neomycin resistance (Southern, P.J. And Berg, P., J. Mol. Appl. Gen., Vol. 1 (1982) 327).

Individual clones were generated by limiting dilution and expanded in the presence of antibiotic. High

expression levels of  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$  were confirmed by flow cytometry using the  $\alpha_v\beta_3$  complex-specific mAb LM609 (Chemicon, Temecula, CA; Proc. Natl. Acad. Sci. USA, Vol. 84 (1987) 6471 and the  $\alpha_v\beta_5$  complex-specific  
5 antibody P1F6 (GIBCO).

#### Osteoclast Adhesion Assay

Rabbit osteoclasts were obtained from the fore and hind  
10 limb bones of 5 day old rabbit pups. Skin and muscle were removed from bones and the bones were pulverized in  $\alpha$  MEM containing 20mM Hepes pH 7.0 and 1% BSA. Clumps of tissue and the released cells were resuspended in  $\alpha$  MEM containing 20mM Hepes pH 7.0 and  
15 1% BSA, clumps of cells were allowed to sediment at unit gravity and the osteoclast containing supernatant was removed and diluted in  $\alpha$  MEM & 20 mM Hepes & 1% BSA.

20 Human osteopontin (OPN) was produced recombinantly as previously described. 96-well plates (Immulon-2, Dynatech, Chantilly, VA) were coated with 5  $\mu$ g/ml OPN in 20 mM Tris, 150 mM NaCl<sub>2</sub>, pH 7.4 (TS) overnight at 4°C. Unreacted sites were blocked with 1%  
25 BSA in TS, 200  $\mu$ l/well) for 2 hours at 37°C. Plates were washed 3 times with TS buffer and 100  $\mu$ l/well of antibodies diluted in  $\alpha$  MEM & 20 mM Hepes & 1% BSA were added. Diluted osteoclasts, 100  $\mu$ l/well, were added to plates and plates were incubated for 60 min at 37°C.

30 Plates were washed 3 times with  $\alpha$  MEM & 1% BSA and 100  $\mu$ l/well of NPP/Tartrate Lysis Buffer (50 mM sodium acetate, 0.5% triton X-100, 0.25 mg/ml p-Nitrophenyl phosphate and 6.7 mM tartrate) was added and incubated for 1 hour at 37°C. Finally, 0.1 N NaOH (50 $\mu$ l/well)  
35 was added and the absorbance at 405 nM determined. The number of cells bound/well were determined by reference to a standard curve generated with known numbers of cells.

## Endothelial Cell Proliferation Assay

Human dermal microvascular endothelial cells were purchased from Clonetics (San Diego, CA) and used for experiments at passages 3-10. Cells were grown in gelatin-coated (1mg/ml) flasks and maintained in MCDB 131 (Gibco), 5% FBS (Hyclone) 100 $\mu$ l/ml mitogen (Biomedical Technologies), 100  $\mu$ l/ml porcine intestinal heparin (Sigma) 2 mM L-glutamine and 100 U/ml penicillin/ 100  $\mu$ g/ml streptomycin (Gibco) at 37°C, 5% CO<sub>2</sub>.

For proliferation experiments, 10 ng/mL human bFGF (R&D Systems) was used in place of mitogen and the final concentration of heparin was reduced to 80  $\mu$ g/mL. Cells were harvested from flasks by trypsinization and plated at 4-6 X 10<sup>3</sup> cells/well (100  $\mu$ l/well) onto 96 well microtiter plates precoated with osteopontin (10  $\mu$ g/ml in Hanks Balanced Salt Solution; 100  $\mu$ l/well). The cells were incubated for 2 hrs at 37°C to allow cell attachment and then monoclonal antibodies (at various concentrations) were added directly to the wells. The plates were returned to the incubator and were grown for ~4 days (~80% confluence) with refeeding every other day. On the final day the cells were supplied fresh medium (not containing antibodies) and proliferation was monitored by adding the REDOX indicator Alamar Blue (Biosource International) in an amount equal to 10% of the culture volume. The plates were incubated for an additional 4-5 hrs and fluorescence at 545/575 nm (excitation/emission) was measured using a PANDEX Fluorescence Concentration Analyzer. The final data was expressed as a percentage of inhibition (comparing antibody treated cells to untreated cells).

Other variations and modifications of this invention will be obvious to those skilled in the art. This

invention is not limited except as set forth in the claims.

We claim:

1. A complex-specific anti- $\alpha_v\beta_3$  integrin monoclonal antibody comprising a monoclonal antibody having the characteristics of any one of the monoclonal antibodies selected from the group consisting of P113-7D6 (HB-12224), P112-4C1 (HB-12225), P113-12A6 (HB-12226), P112-11D2 (HB-12227), P112-10D4 (HB-12228) or P113-1F3 (HB-12229).
2. A pharmaceutical composition suitable for use in humans comprising the monoclonal antibody of Claim 1.
3. A monoclonal antibody having the characteristics of monoclonal antibody P113-7D6 (HB-12224).
4. A monoclonal antibody having the characteristics of monoclonal antibody P112-4C1 (HB-12225).
5. A monoclonal antibody having the characteristics of monoclonal antibody P113-12A6 (HB-12226).
6. A monoclonal antibody having the characteristics of monoclonal antibody P112-11D2 (HB-12227).
7. A monoclonal antibody having the characteristics of monoclonal antibody P112-10D4 (HB-12228).

8. A monoclonal antibody having the characteristics of monoclonal antibody P113-1F3 (HB-12229).

9. A method for treating conditions mediated by  $\alpha_v\beta_3$  integrin in a mammal in need of such treatment comprising administering an effective  $\alpha_v\beta_3$  integrin inhibiting amount of an antibody of Claim 1.

10. A method according to Claim 9 wherein the condition is osteoporosis.

11. A method according to Claim 9 wherein the condition is humoral hypercalcemia of malignancy.

12. A method according to Claim 9 wherein the condition is mediated by smooth muscle cell migration.

13. The method of Claim 12, wherein the condition is atherosclerosis.

14. A method according to Claim 9 wherein the condition is restenosis.

15. A method according to Claim 9 wherein the condition is selected from the group consisting of ocular neovascularization, macular degeneration and diabetic retinopathy.



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16. A method according to Claim 9 wherein the condition is psoriasis.

17. A method according to Claim 9 wherein the condition is rheumatoid arthritis.

18. An immunodiagnostic diagnostic kit comprising a monoclonal antibody having the characteristics of any one of the monoclonal antibodies selected from the group consisting of P113-7D6 (HB-12224), P112-4C1 (HB-12225), P113-12A6 (HB-12226), P112-11D2 (HB-12227), P112-10D4 (HB-12228) and P113-1F3 (HB-12229).

19. A method for determining the presence of a condition mediated by  $\alpha_v\beta_3$  integrin in a mammal comprising the steps of:  
reacting an  $\alpha_v\beta_3$  complex-specific antibody with a tissue sample from the mammal, washing away complex-specific antibody that is not bound to the tissue sample;  
reacting a second antibody with the tissue sample, the second antibody having a reporter molecule attached thereto; washing away second antibody that is not bound to the tissue sample and detecting the presence reporter molecules in the sample.

20. The method of Claim 19, wherein the presence of  $\alpha_v\beta_3$  bearing neoplasm or tumor related vascular bed is detected by NMR or immunoscintigraphy.

21. A method according to Claim 9 wherein the condition is osteoarthritis.

22. A method for delivering a cytotoxic or cytostatic compound to cells having an  $\alpha_v\beta_3$ -integrin, comprising contacting the cells with a monoclonal antibody of Claim 1, or fragment thereof containing the paratope, wherein the antibody or fragment thereof is conjugated with the cytotoxic or cytostatic compound.

23. The method of Claim 22, wherein  $\alpha_v\beta_3$ -integrin-bearing cells are killed.

24. The method of Claim 22, wherein the growth of  $\alpha_v\beta_3$ -integrin-bearing cells is inhibited.

25. The method of Claim 22, wherein the monoclonal antibody or fragment thereof is conjugated to a cytotoxic or cytostatic agent selected from the group consisting of doxorubicin, daunomycin, methotrexate, cisplatin, bleomycin, vinblastine, mitomycin C, idarubicin, morpholinodoxorubicin, melphalan, cytosine arabinoside, 5-fluorouracil, and neocarzinostatin.

26. A method for delivering nucleic acid to cells having an  $\alpha_v\beta_3$ -integrin, comprising contacting the cells with a monoclonal antibody of Claim 1, or fragment thereof containing the paratope, wherein the antibody or fragment thereof is conjugated with a nucleic acid.

27. The method of Claim 26, wherein the nucleic acid is an anti-sense oligonucleotide, the oligonucleotide being optionally modified to be nuclease resistant.

28. The method of Claim 26, wherein the oligonucleotide is 5'-GTGCCGGGGTCTTCGGGC-3'.

29. A method for delivering a gene to cells having an  $\alpha_v\beta_3$ -integrin, comprising contacting the cells with a monoclonal antibody of Claim 1, or fragment thereof containing the paratope, wherein the antibody or fragment thereof is conjugated to the cDNA of the gene.

30. The method of Claim 29, wherein the cDNA encodes for the HIV-1 thymidine kinase gene.

31. A method for delivering a radioisotope to cells having an  $\alpha_v\beta_3$ -integrin, comprising contacting the cells with a monoclonal antibody of Claim 1, or fragment thereof containing the paratope, wherein the antibody or fragment thereof is conjugated to the radioisotope.

32. The method of Claim 31 wherein the radioisotope is selected from the group consisting of  $^{64}\text{Cu}$ ,  $^{67}\text{Cu}$ ,  $^{90}\text{Y}$ ,  $^{99\text{m}}\text{Tc}$ ,  $^{103}\text{Pd}$ ,  $^{105}\text{Rh}$ ,  $^{109}\text{Pd}$ ,  $^{111}\text{In}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{153}\text{Sm}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{192}\text{Ir}$ ,  $^{198}\text{Au}$ ,  $^{203}\text{Pb}$ ,  $^{212}\text{Pb}$ ,  $^{212}\text{Bi}$  and  $^{241}\text{Am}$ .

33. A method for delivering a bacterial toxin to cells having an  $\alpha_v\beta_3$ -integrin, comprising contacting the cells with a monoclonal antibody of Claim 1, or fragment thereof containing the paratope, wherein the antibody or fragment thereof is conjugated to the bacterial toxin.

34. The method of Claim 33 wherein the bacterial toxin is selected from the group consisting of Pseudomonas exotoxin, diphtheria toxin and shiga toxin.

35. A method for delivering a plant toxin to cells having an  $\alpha_v\beta_3$ -integrin, comprising contacting the cells with a monoclonal antibody of Claim 1, or fragment thereof containing the paratope, wherein the antibody or fragment thereof is conjugated to the plant toxin.

36. The method of Claim 35 wherein the plant toxin is selected from the group consisting of ricin, abrin, modeccin, gelonin, pokeweed antiviral protein (PAP),  $\alpha$ -amanitin, ribosome inhibiting protein (RIP), barley RIP, wheat RIP, corn RIP and flax RIP.

37. The hybridoma cell capable of making monoclonal antibody having the characteristics of monoclonal antibody P113-7D6 (HB-12224).

38. The hybridoma cell capable of making monoclonal antibody having the characteristics of monoclonal antibody P112-4C1 (HB-12225).

39. The hybridoma cell capable of making monoclonal antibody having the characteristics of monoclonal antibody P113-12A6 (HB-12226).

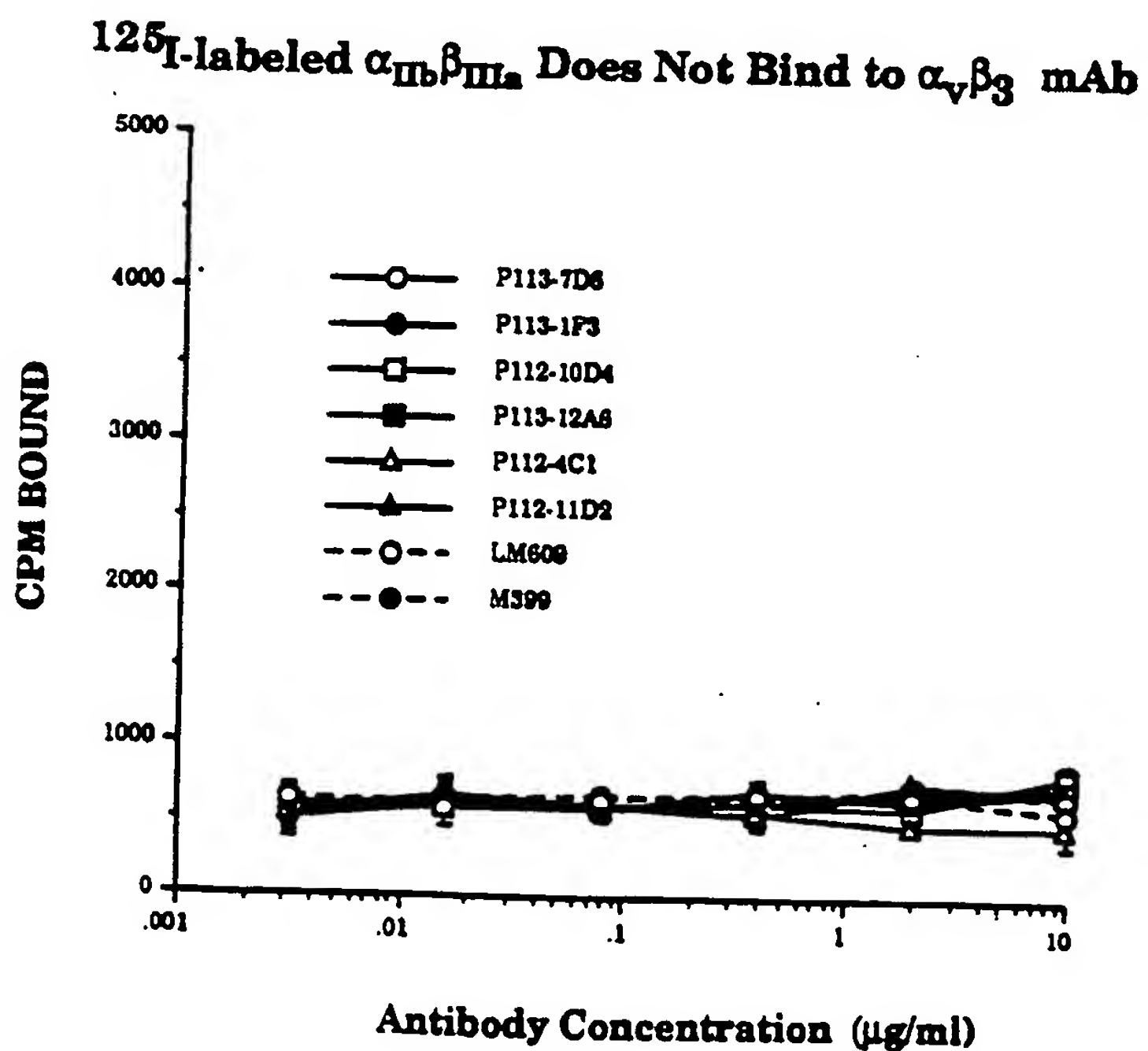
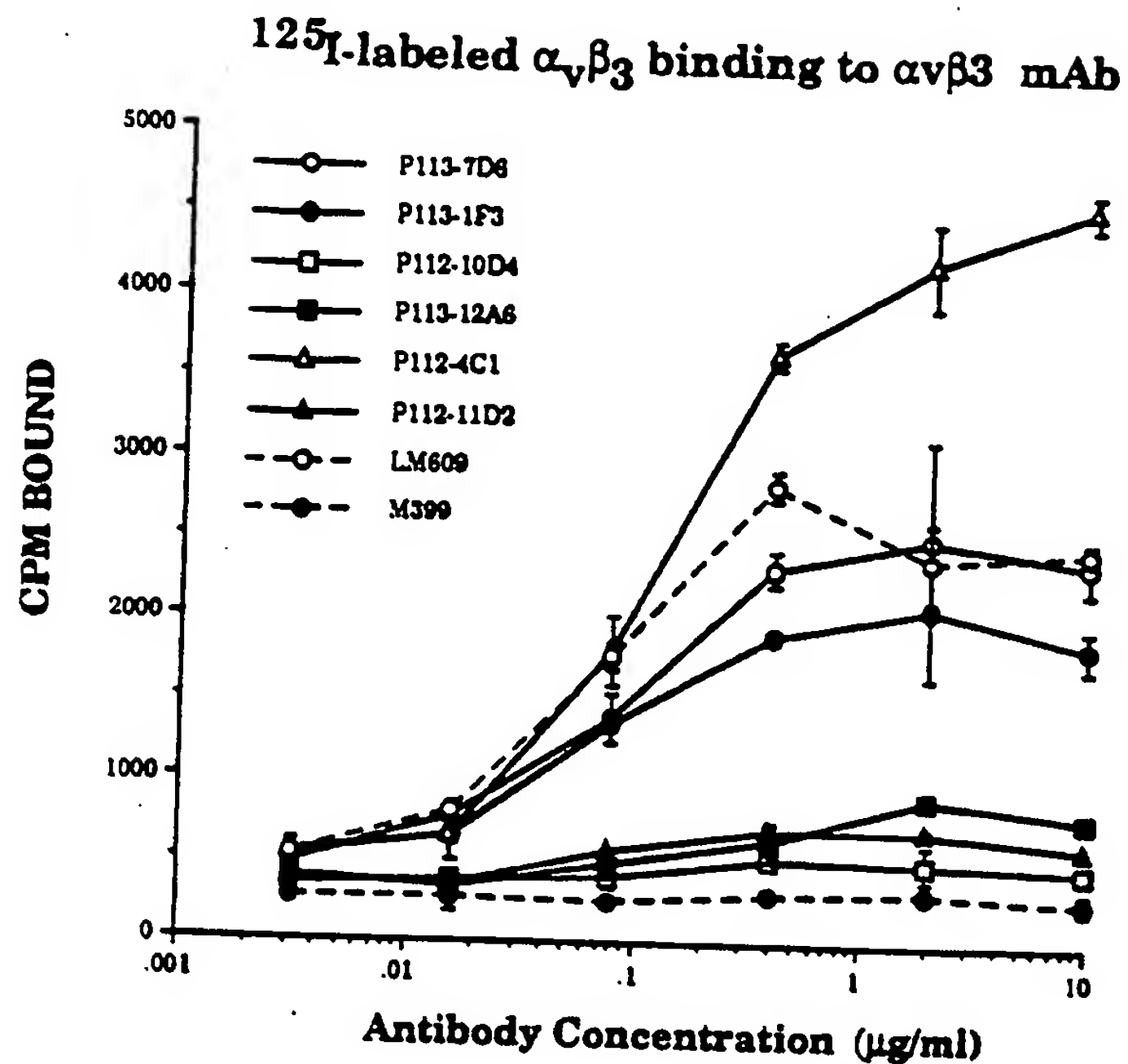
40. The hybridoma cell capable of making monoclonal antibody having the characteristics of monoclonal antibody P112-11D2 (HB-12227).

41. The hybridoma cell capable of making monoclonal antibody having the characteristics of monoclonal antibody P112-10D4 (HB-12228).

42. The hybridoma cell capable of making monoclonal antibody having the characteristics of monoclonal antibody P113-1F3 (HB-12229).

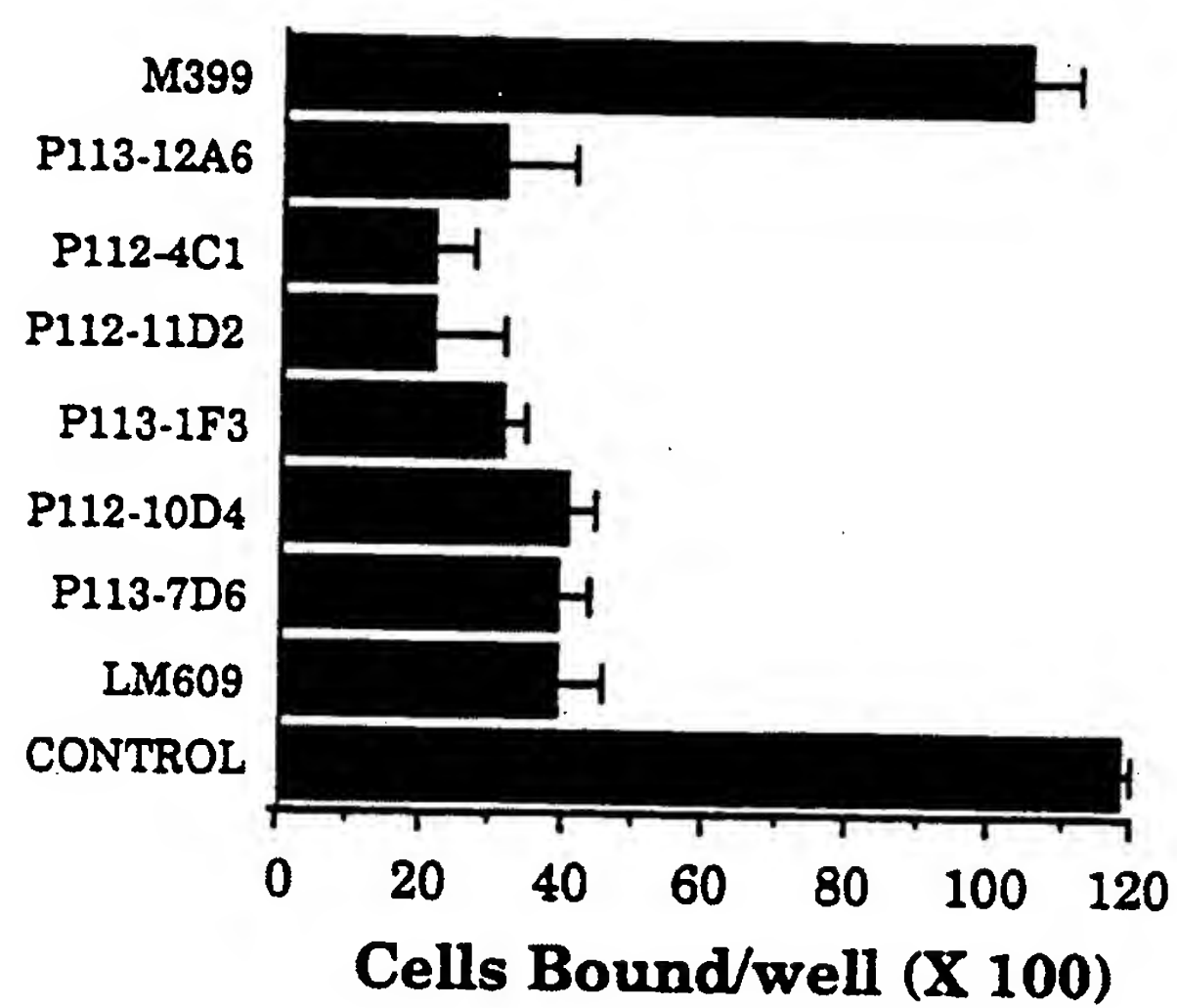
1/8

Fig 1





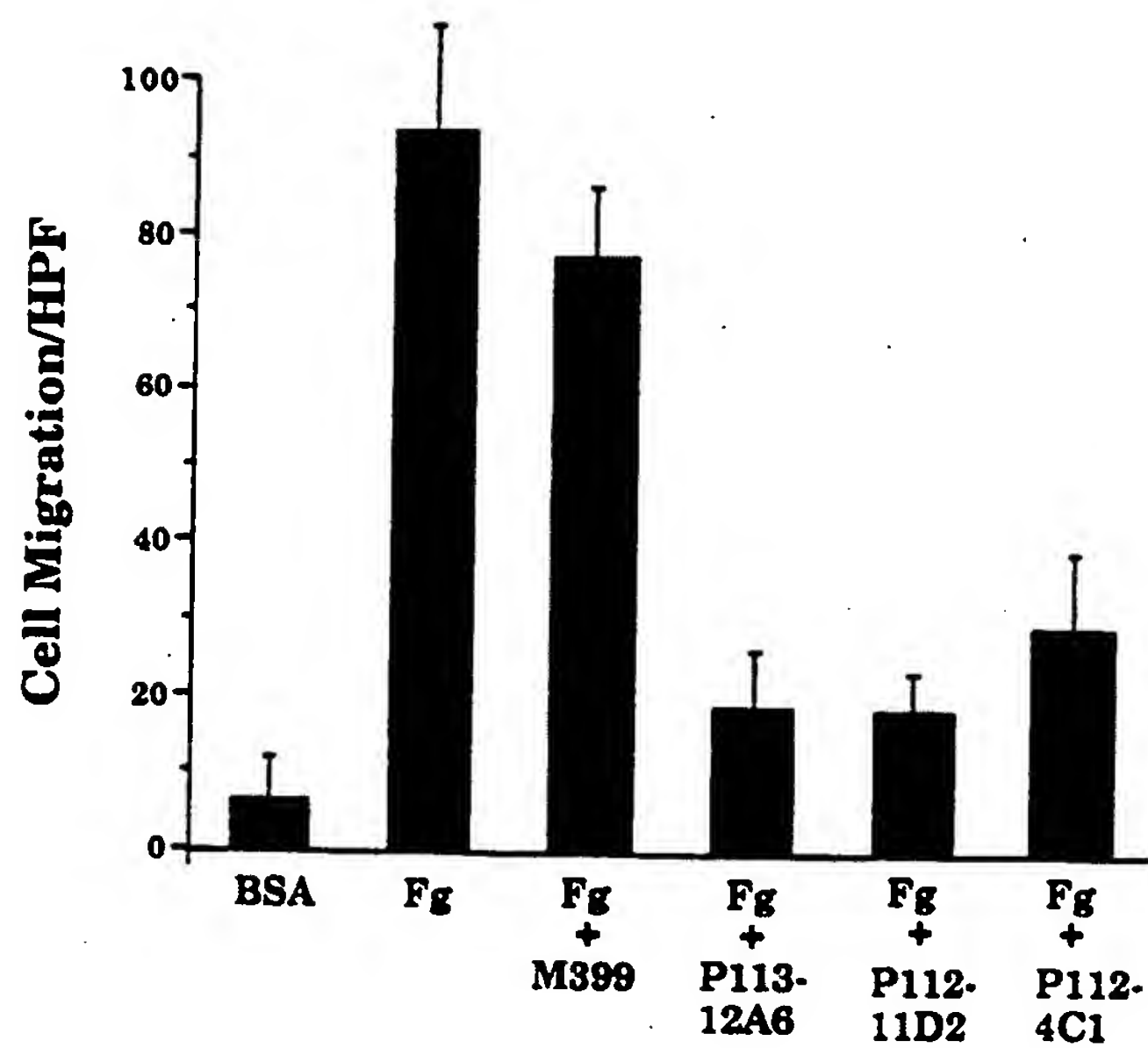
2/8

**Fig 2** **$\alpha$ v $\beta$ 3 Antibodies Inhibit M21 Cell Binding to Fibrinogen**

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**Fig 3** **$\alpha v\beta 3$  Antibodies Inhibit M21 Cell Migration**

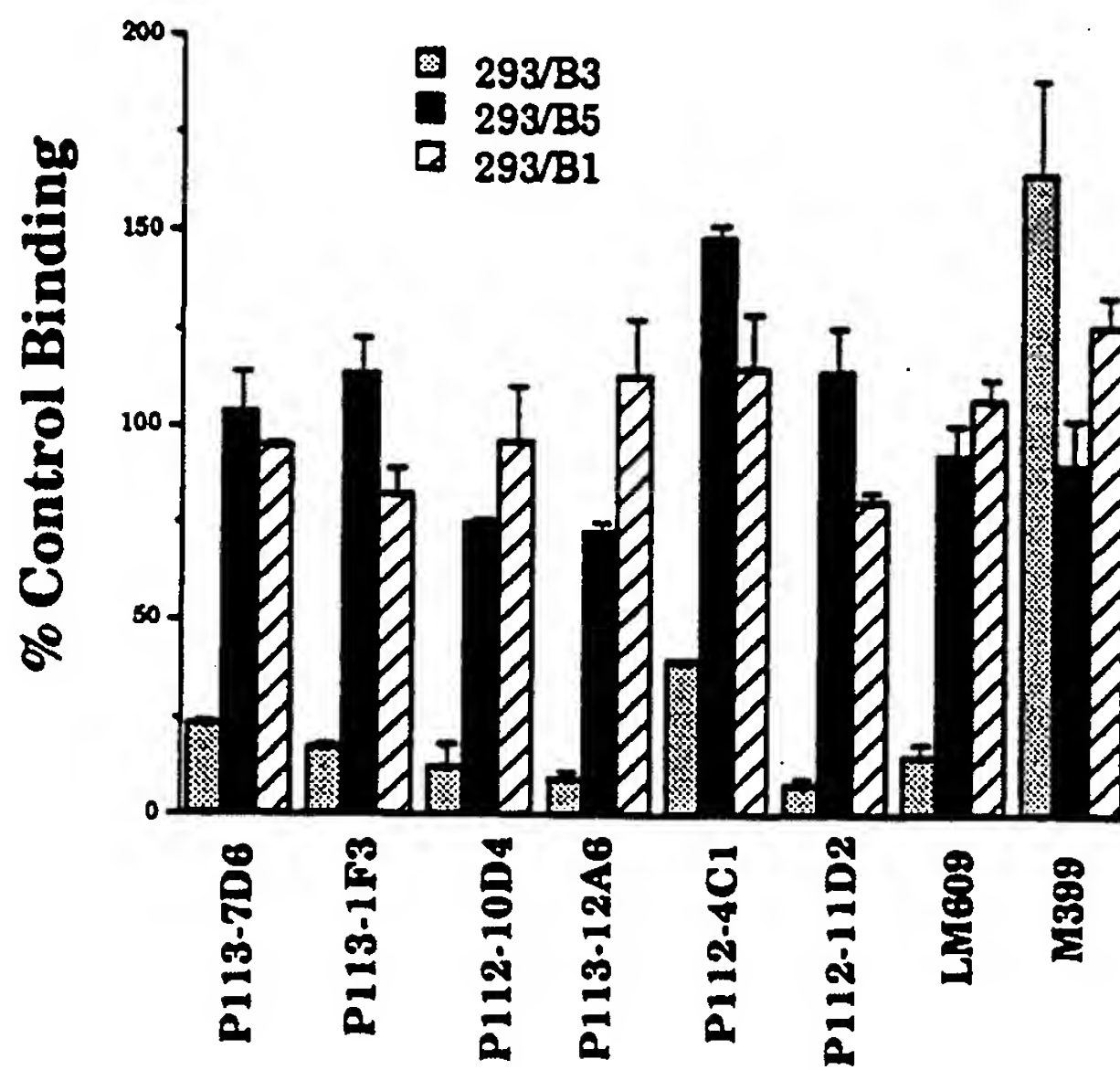
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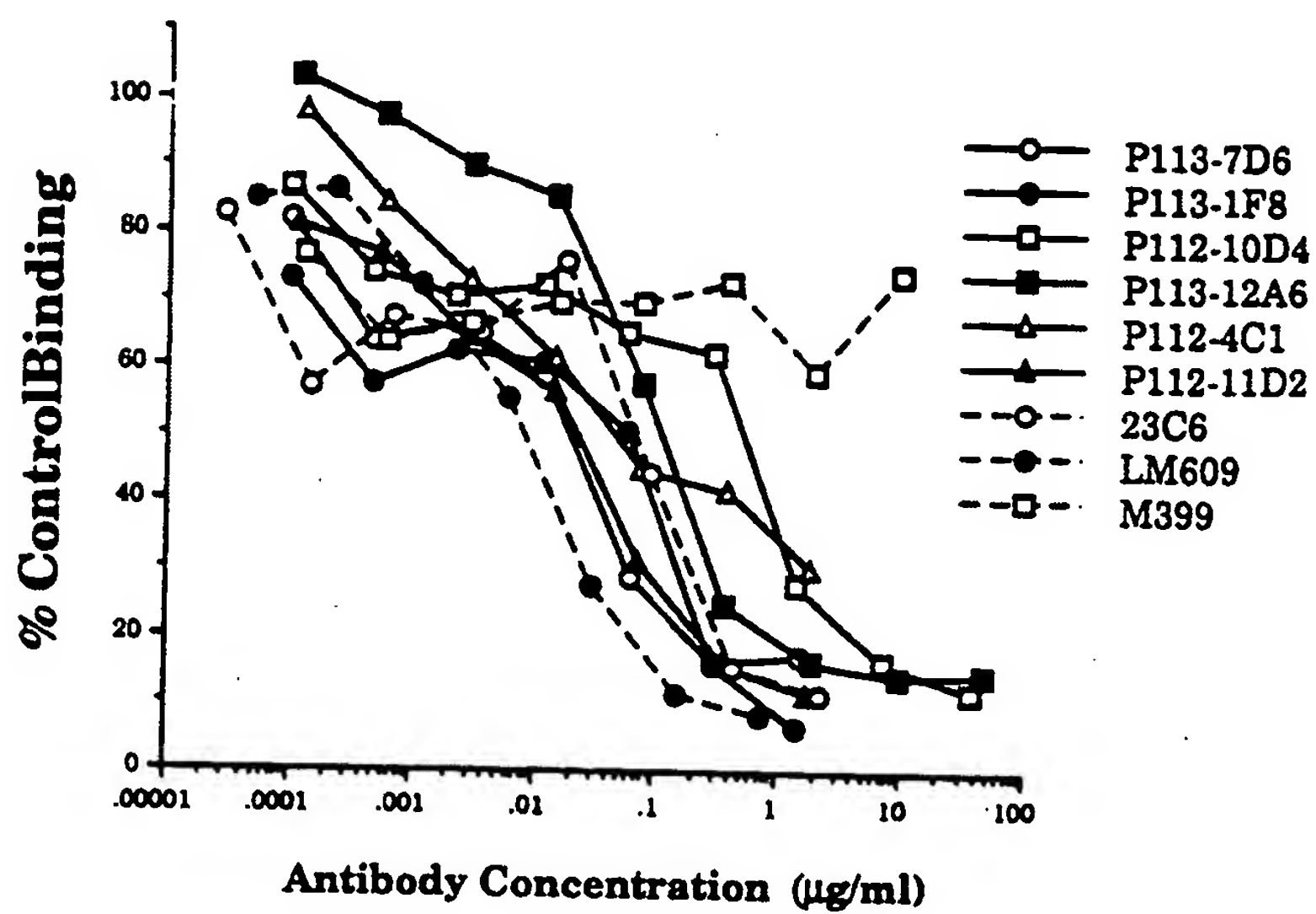
**Fig 4**

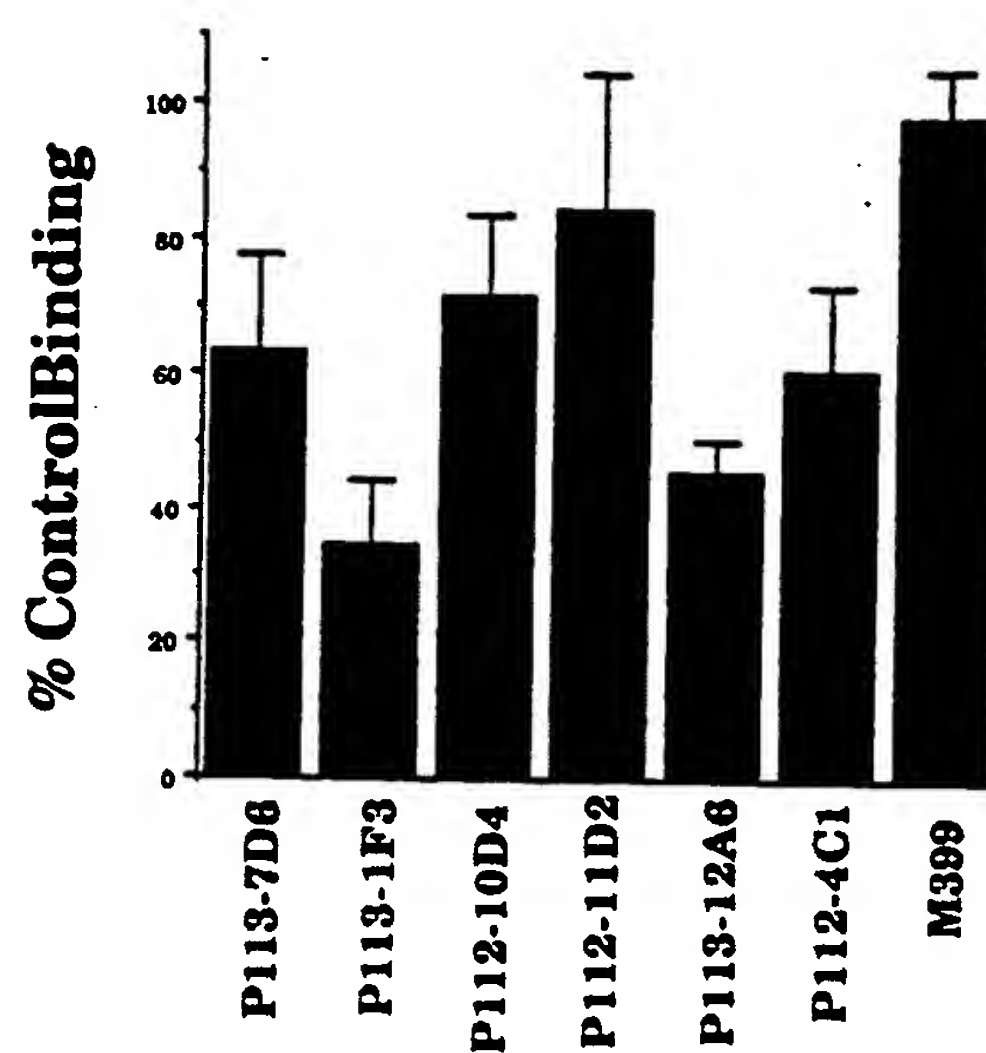
**$\alpha_v\beta_3$ Antibodies Inhibit 293/ $\beta_3$ - But Not  
293/ $\beta_5$ - or 293/ $\beta_1$ -Cell Adhesion to Vitronectin**



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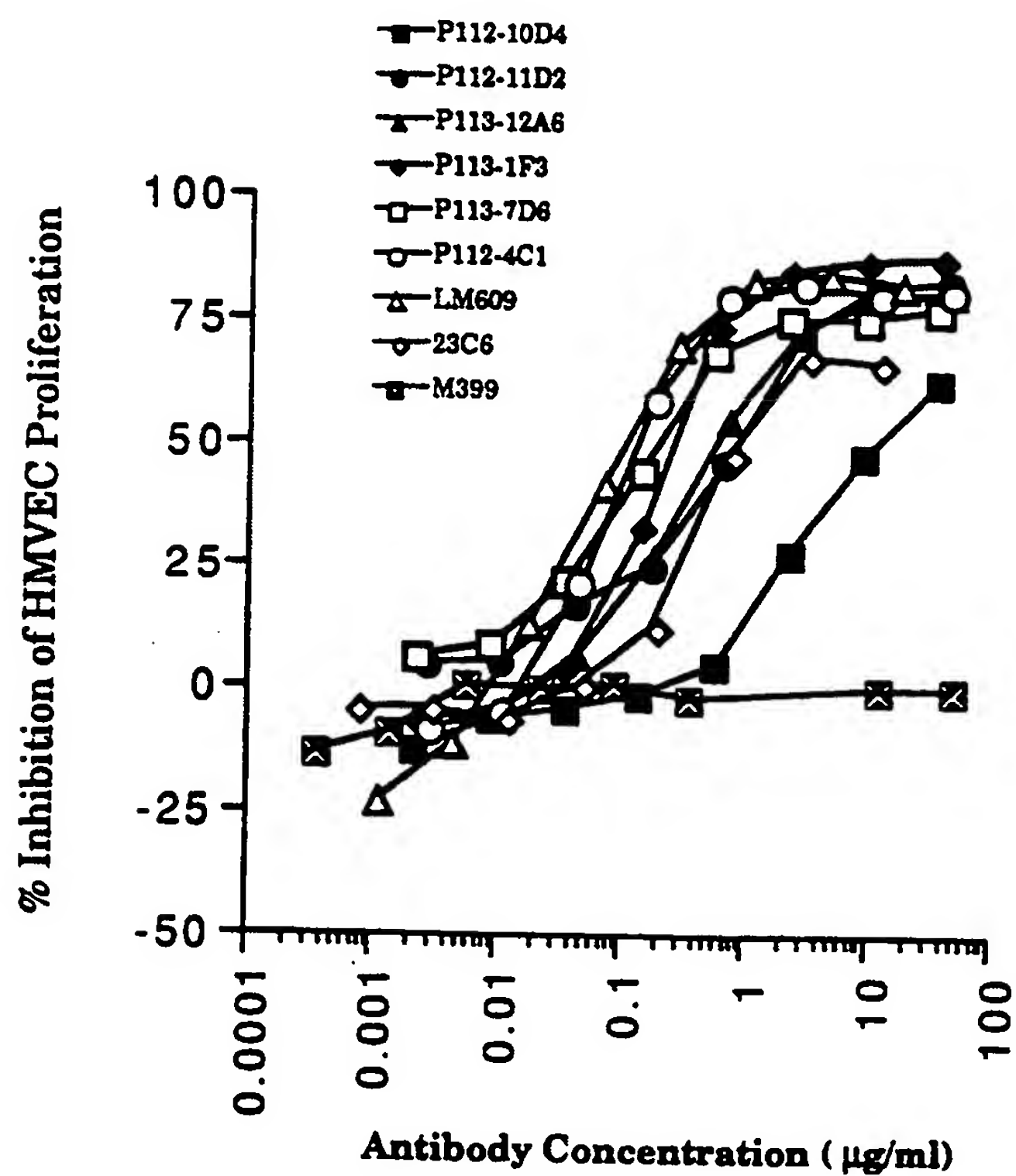
SUBSTITUTE SHEET (RULE 26)

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Fig 5 **$\alpha_v\beta_3$  Antibodies Inhibit 293/ $\beta_3$  Cell Adhesion  
to Vitronectin**

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**Fig 6** **$\alpha$ v $\beta$ 3 Antibodies Inhibit Rabbit Osteoclast  
Adhesion to Osteopontin**

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Fig 7 **$\alpha$ v $\beta$ 3 Antibodies Inhibit Endothelial Cell Proliferation**

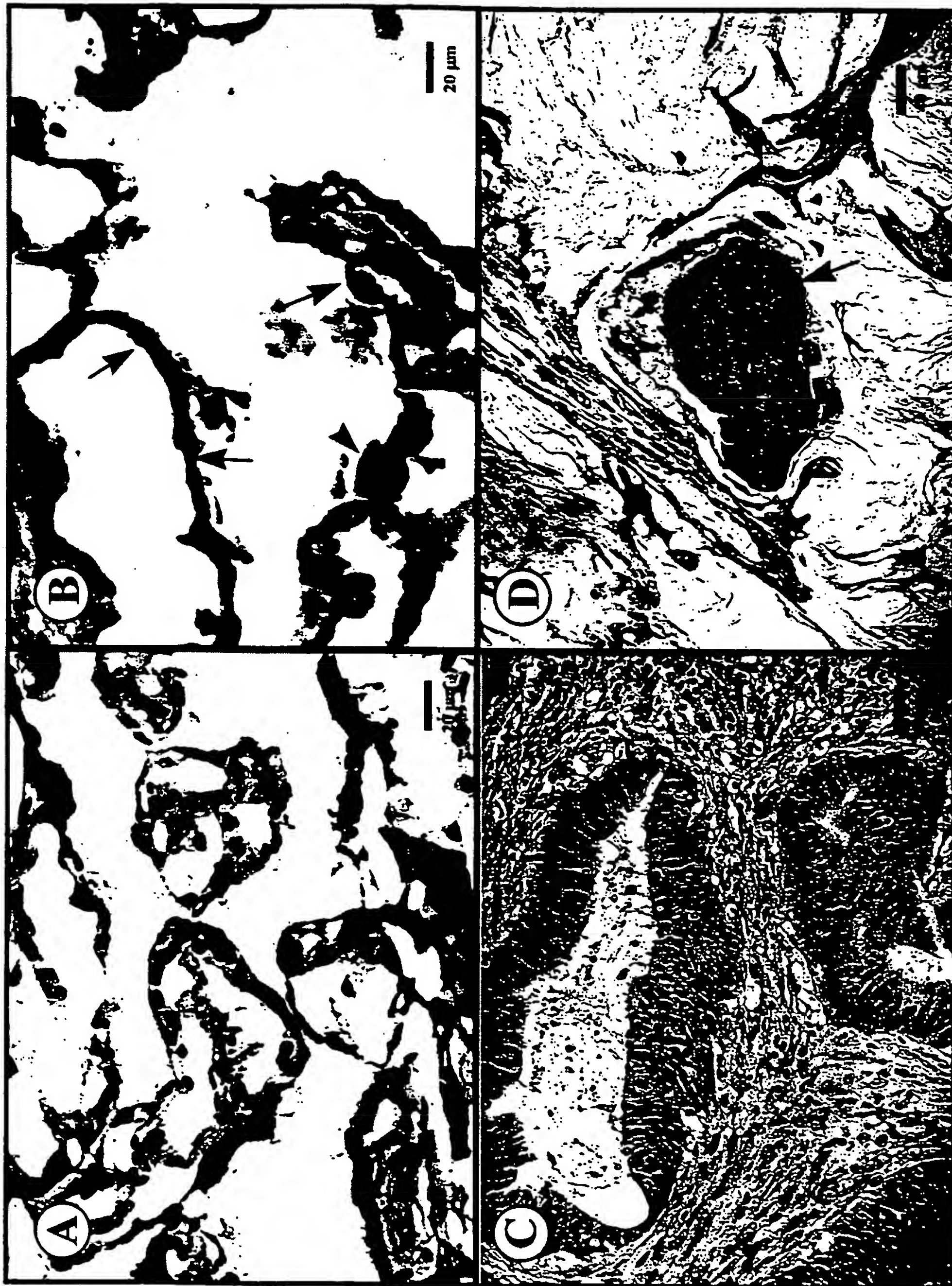
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FIGURE 8



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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/06867

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NICKOLS ET AL.: "Antiangiogenic and anticancer activities of antagonists of integrin avb3" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 38, no. A1389, March 1997, page 206 XP002071773 see the whole document	1,2,6,9, 11,40
Y		22-28, 35,36
A		3-5,7,8, 37-39, 41,42
	--- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance  
 "E" earlier document but published on or after the international filing date  
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.  
 "&" document member of the same patent family

Date of the actual completion of the international search

16 July 1998

Date of mailing of the international search report

03/08/1998

Name and mailing address of the ISA

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 Fax: (+31-70) 340-3016

Authorized officer

Covone, M

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/06867

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 89 05155 A (SCRIPPS CLINIC RES) 15 June 1989</p> <p>see page 4, line 15 - line 22  see page 5, line 16 - line 32  see page 21, line 13 - line 16  see page 24, line 23 - line 29  see page 38 section 5</p> <p>---</p>	<p>1,2,  18-20,  31,32</p>
X	<p>WO 95 25543 A (SCRIPPS RESEARCH INST) 28 September 1995</p> <p>see examples 2,7E1,8  see claims</p> <p>---</p>	<p>1,2,9,  10,12-18</p>
X	<p>WO 93 20229 A (GENENTECH INC ;KIM KYUNG JIN (US)) 14 October 1993</p> <p>see page 7, line 9 - page 10, line 29  see page 14, line 1 - line 8</p> <p>---</p>	<p>1,2,9,  10,  18-24,  31-34</p>
Y	<p>WO 90 07936 A (CHIRON CORP) 26 July 1990</p> <p>see page 11, line 10  see page 11, line 30 - line 32  see page 15, line 15 - line 28</p> <p>---</p>	<p>22-25,  35,36</p>
A		<p>29,30</p>
Y	<p>EP 0 653 439 A (HOECHST AG) 17 May 1995</p> <p>see page 2, line 1 - line 18  see page 8, line 43 - line 44</p> <p>-----</p>	<p>26-28</p>

## INTERNATIONAL SEARCH REPORT

international application No.

PCT/US 98/ 06867

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 9-17, 21 (all complete) 22-36 (all partially, as far as in vivo treatment is concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-2 (partially), 3 (completely), 9-36 (partially),  
37 (completely)

Anti-alfavbeta3 complex specific monoclonal antibody  
P113-7D6, its hybridoma, and therapeutic and diagnostic uses  
thereof.

2. Claims: 1-2 (partially), 4 (completely), 9-36 (partially),  
38 (completely)

Anti-alfavbeta3 complex specific monoclonal antibody  
P112-4C1, its hybridoma and therapeutic and diagnostic uses  
thereof.

3. Claims: 1-2 (partially), 5 (completely), 9-36 (partially),  
39 (completely)

Anti-alfavbeta3 complex specific monoclonal antibodies  
P113-12A6, its hybridoma and therapeutic and diagnostic uses  
thereof

4. Claims: 1-2 (partially), 6 (completely) 9-36 (partially)  
40 (completely)

Anti-alfavbeta3 complex specific monoclonal antibody  
P112-11D2, its hybridoma and therapeutic and diagnostic uses  
thereof

5. Claims: 1-2 (partially), 7 (completely), 9-36 (partially),  
41 (completely)

Anti-alfavbeta3 complex specific monoclonal antibody  
P112-10D4, its hybridoma and therapeutic and diagnostic uses  
thereof

6. Claims: 1-2 (partially), 8 (completely), 9-36 (partially),  
42 (completely)

Anti-alfavbeta3 complex specific monoclonal antibody  
P113-1F3, its hybridoma and therapeutic and diagnostic uses  
thereof.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. National Application No

PCT/US 98/06867

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8905155 A	15-06-1989	AU 2807189 A DK 355589 A EP 0341303 A FI 893475 A JP 2502156 T	05-07-1989 18-09-1989 15-11-1989 18-07-1989 19-07-1990
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EP 0653439 A	17-05-1995	DE 4338704 A AU 7779994 A CA 2135591 A JP 7194385 A NZ 264894 A	18-05-1995 18-05-1995 13-05-1995 01-08-1995 27-07-1997